REGULATION OF THE ENZYME SECRETION BY THE PANCREAS

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CONTENTS

The pancreas secretes a juice, consisting of water, electrolytes and digestive enzymes (or their zymogens), which plays an essential role in the digestion of food in the duodenum. The part of the pancreas responsible for the formation and secretion of this fluid, called the exocrine pancreas, consists mainly of acinar cells. These cells synthesize, store and secrete the digestive enzymes. The enzyme secretion process can be stimulated by acetylcholine or by the hormone pancreozymin, without their penetration into the cells. The interaction between the stimulant and its receptor on the plasma membrane triggers an intracellular process, which results in secretion of enzymes. This intracellular process is called stimulus-secretion coupling.

It was already known that the C-terminal part of pancreozymin, and in particular the tryptophan residue in position 4 from the C-terminal end, is essential for the hormone-receptor interaction. We have now investigated which property of tryptophan is important for this interaction.

For various substances there are indications that they are involved as second messengers in the enzyme secretion process. To obtain more information about their role we tried to introduce these substances in the cytoplasm by means of liposomes.

The fact that pancreozymin stimulates the adenylate cyclase activity in the acinar cell indicates that cyclic AMP may be involved in enzyme secretion. However, previous studies have shown little or no rise in the pancreatic cyclic AMP content by application of pancreozymin. We have studied this discrepancy in relationship to the extracellular calcium concentration.

While the role of cyclic AMP as a second messenger is still questionable, there are strong indications that a rise in cytoplasmic calcium concentration is an important step in stimulus-secretion coupling. In order to obtain further information about the role of

calcium in enzyme secretion, the calcium movements in the pancreas have been studied under resting and stimulated conditions.

Finally, the role of sodium as a second messenger has been studied by observing the effects of a low sodium medium and of ouabain on the stimulation of enzyme secretion and on calcium fluxes. The significance of the results for our understanding of the mechanism of the stimulus-secretion coupling in the exocrine pancreas is discussed.

CHAPTER I.

INTRODUCTION

1.1. Structure and function of the exocrine pancreas

The pancreas has an endocrine as well as an exocrine function. The endocrine tissue accounts for only about 2% of the total tissue volume and consists of small clusters of cells, the islets of Langerhans, dispersed throughout the exocrine tissue. These cells synthesize and secrete the hormones insulin and glucagon, which regulate the blood glucose level, and also somatostatin, which interacts with insulin and glucagon and is in addition involved in the exocrine pancreatic secretion.

The exocrine part of the pancreas secretes pancreatic juice, a fluid containing electrolytes and digestive enzymes. This fluid plays a crucial role in the digestion of food in the duodenum. The major part (ca 90%) of the exocrine tissue consists of cells with a pyramidal shape, the acinar cells. These cells are characterized by a highly developed endoplasmic reticulum and Golgi apparatus and contain a large number of zymogen granules, situated at the apical side of the cell. The functional unit of the pancreas, the acinus, is formed by several acinar cells situated around a common lumen. A few cells in the acinus are much smaller than the acinar cells and do not contain zymogen granules. They are called the centro-acinar cells and they border on a ductule connecting the acinar lumen with the duct system. This duct system is formed by numerous small ducts converging to larger ducts, finally resulting in a single main duct which ends in the duodenum. The ducts are lined by ductular cells, small epithelial cells which do not contain zymogen granules. Based on their morphology, it can be assumed that the centro-acinar and ductular cells have the same function. The morphological aspects of the pancreas have been elucidated by various light microscopic (Bloom and Fawcett, 1975) and electron microscopic studies (Ekholm

et al., 1962a, 1962b; Palade et al., 1962; Sjöstrand, 1962; Kern and Ferner, 1971).

When the pancreas is in the resting state, there is only a slight secretion of fluid and digestive enzymes. When the pancreas is stimulated, e.g. by the intake of a meal, the secretion of enzymes, fluid and electrolytes greatly increases. The enzymes, originating from the acinar cells, are transported via the duct system to the duodenum, where they contribute to the digestion of macromolecular components of the food.

The regulation of the pancreatic secretion takes place in three phases. The mere observation of food causes a neural stimulation of the pancreas. The extension of the stomach volume after food intake stimulates the pancreatic secretion via a vago-vagal and local cholinergic pathway. The entrance of food in the intestine causes a release of the hormonal stimulants secretin and pancreozymin (Harper, 1967).

1.2. Secretion of fluid and electrolytes

1.2.1. Electrolyte composition of pancreatic fluid

The pancreas secretes a fluid, which is isosmotic with the extracellular fluid at all flow rates (Case et al., 1968; Case and Scratcherd, 1974). The major cations in the pancreatic fluid are sodium and potassium. Their concentration is about the same as in the plasma or perfusion medium (Ball and Johnston, 1930; Dreiling and Janowitz, 1956; Rutten, 1974; Bonting et al., 1980). Under non-stimulated condition the calcium and magnesium concentrations in the secreted fluid are lower than in the extracellular fluid. When the pancreas is stimulated by cholinergic agents or pancreozymin the concentration of these ions increases. This effect can be partly ascribed to the contribution of protein-bound calcium and magnesium (Argent et al., 1973; Schreurs et al., 1975), but partly also to an increase in the permeability of a paracellular transport route (Schreurs et al., 1975).

The major anions in the pancreatic fluid are bicarbonate and chloride. In general the concentration of bicarbonate is higher and that of chloride lower than in the blood plasma. In several species it is found that with increasing flow rate the concentration of bicarbonate rises and that of chloride decreases, while the sum of both concentrations remains constant (Janowitz, 1967; Case et al., 1968). This observation suggests that there is an exchange of bicarbonate in the secreted fluid with chloride during the passage through the ducts (Case et al., 1968).

1.2.2. Origin of fluid and electrolytes

There are several indications that the ductular cells are involved in the secretion of fluid and electrolytes. Selective damage of the ductular cells by alloxan decreases the stimulatory effect of secretin on the pancreatic fluid secretion (Grossman and Ivy, 1946). When, however, the acinar cells are destroyed by ethionine in the diet (Kaiser and Grossman, 1954) or by keeping rats on a copper-deficient diet containing penicillamin (Fölsch and Creutzfeldt, 1977), the pancreas is still able to secrete fluid and electrolytes.

These experiments do not exclude the possibility that acinar cells are also involved in the fluid and electrolyte secretion. Indeed, Dockray (1972), Sewell and Young (1975) and Petersen and Ueda (1977) have provided evidence for fluid secretion by the acinar cells. The contribution of this primary secretion to the overall fluid secretion is large in the rat pancreas and small in the cat and rabbit pancreas.

1.2.3. Regulation of fluid and electrolyte secretion

The pancreatic fluid secretion is mainly regulated by a hormonal mechanism and not by neural control. Entrance of the acid food mass from the stomach into the duodenum causes a release of the hormones secretin and pancreozymin from the endocrine cells of the duodenal mucosa (Wang and Grossman, 1951; Bussolati, 1971). These hormones reach the pancreas via the blood circulation. Secretin mainly stimulates the fluid secretion by the ductular cells (Schulz et al.,

1969; Swanson and Solomon, 1973). In some species like rat and dog pancreozymin also stimulates the fluid secretion (Henriksen, 1968; Dockray, 1972; Sewell and Young, 1975), probably the fraction originating from the acinar cells (Petersen and Ueda, 1977).

In the last decade two other polypeptide hormones have been discovered, which may be involved in pancreatic secretion. Said and Mutt (1970) isolated from the small intestine of the pig the "vasoactive intestinal polypeptide" (VIP), which resembles secretin in structure (Said and Mutt, 1972; Bodanszky, 1973) and biological activity (Makhlouf, 1974). It is uncertain whether VIP plays a role in the regulation of fluid secretion under physiological conditions, for the concentration needed to cause an increased fluid secretion is very high compared to that of secretin (Said and Mutt, 1972; Konturek, 1976a; Domschke, 1977; Jensen et al., 1978).

Arimura et al. (1975) have shown that the polypeptide somatostatin is synthesized in brain, stomach and pancreas. This suggests that somatostatin may be involved in the regulation of the pancreatic secretion. However, the effect of somatostatin on the fluid secretion varies somewhat. In man it inhibits the stimulated fluid secretion (Hanssen et al., 1977). The same effect has been observed in the dog by Boden et al. (1975), Wilson et al. (1977) and Konturek et al. (1978), but Kayasseh et al. (1978) do not find a significant effect. In pig (Polak et al., 1975), cat (Albinus et al., 1977) and rat (Fölsch et al., 1978) somatostatin seems to have no effect on the stimulated fluid secretion. There are indications that the inhibiting effect of somatostatin on the fluid secretion is caused by an inhibition of the acid-induced release of secretin from the duodenum (Boden et al., 1975; Hanssen et al., 1977). So the physiological role of somatostatin could be the control of the fluid secretion on a secondary level.

1.2.4. Stimulus-secretion coupling

As mentioned before, the pancreatic fluid secretion can be increased by hormonal stimulation. The hormone arrives at the ductular cell on the basal side and is probably unable to enter the cell. So

the hormone-receptor interaction at the basal membrane must activate an intracellular process,which finally results in the fluid secretion. This intracellular process is called stimulus-secretion coupling. In several tissues cAMP is as "second messenger" involved in this type of process (see Robison et al., 1971). There are strong indications that cAMP is also involved in the secretin-induced fluid secretion. Upon addition of secretin there is an activation of the enzyme adenylate cyclase, which is responsible for cAMP formation (Rutten et al., 1972; Kempen et al., 1974). Milutinovic et al. (1977) showed that there is adenylate cyclase activity both in ductular and acinar cells, but that the enzyme in the ductular cells is much more sensitive to secretin.

In addition, it has been shown that secretin does indeed cause a rise in the cAMP-level in the pancreas (Case et al., 1972; Benz et al., 1972; Robberecht et al., 1974; Kempen et al., 1977a). A role for cAMP in the secretin-evoked fluid secretion is also supported by the fact that theophylline, which prevents the breakdown of cAMP, stimulates fluid secretion (Case and Scratcherd, 1972) and that inhibition of the adenylate cyclase activity by alloxan also inhibits fluid secretion (Scratcherd, 1974; Bonting et al., 1977). How the increase in the cAMP content of the ductular cells leads to fluid secretion is still unclear.

Much less is known about the fluid secretion process of the acinar cells. Pancreozymin, which stimulates this primary fluid secretion, activates the adenylate cyclase activity in the acinar cells, but this causes no (Robberecht et al., 1974; Albano et al., 1976) or only a small rise in the cyclic AMP content (Kempen et al., 1977a). Besides stimulating adenylate cyclase, pancreozymin also increases the cytoplasmic calcium concentration (see section 1.3.5). Whether these two phenomena are involved in fluid secretion is unknown.

1.2.5. Transport processes involved in fluid secretion

Reduction of the extracellular sodium concentration causes a decrease in the flow rate (Ridderstap and Bonting, 1969; Rothman and

Brooks, 1965; Case et al., 1968; Swanson and Solomon, 1975; Jansen, 1980). Several investigators have also found that addition of ouabain which inhibits the Na^+-K^+ATPase activity, lowers the fluid secretion (Ridderstap and Bonting, 1969; Guelrud et al., 1972; Case and Scratcherd, 1974; Swanson and Solomon, 1975). These findings suggest that sodium transport from plasma to lumen by means of Na^+-K^+ATPase is involved in fluid secretion.

Potassium ions do not seem to play a primary role in the pancreatic fluid secretion. Incubation in potassium-free medium causes a reduction of fluid secretion, but the fact that rubidium can replace potassium suggests that the reduction can be explained by the inhibition of Na^+-K^+ATPase in potassium-free medium.

There are some indications that bicarbonate transport is important for the fluid secretion. The fluid secretion rate depends on the bicarbonate concentration of the extracellular medium (Case et al., 1970; Schulz et al., 1971; Swanson and Solomon, 1975). Another argument is that radioactive bicarbonate, added to the external medium, appears very rapidly in the secreted fluid (Case et al., 1970). However, the observation that other weak acids, like acetate, propionate, butyrate and formate (Swanson and Solomon, 1975; Case et al., 1979) and even non-carboxylic weak acids like sulfamerazine and glycodiazine (Schulz et al., 1971a, 1971b) can substitute for bicarbonate, suggests that not just bicarbonate, but any anion of a weak acid can satisfy the requirements for fluid secretion.

Replacing chloride by bromide or nitrate has no effect on fluid secretion, but replacing chloride by the impermeable isethionate inhibits fluid secretion (Case et al., 1979). This indicates that permeable anions are necessary for the fluid secretion. Fig. 1.1 shows a model for the fluid secretion process, both in ductular and acinar cells, recently developed by Jansen (1980).

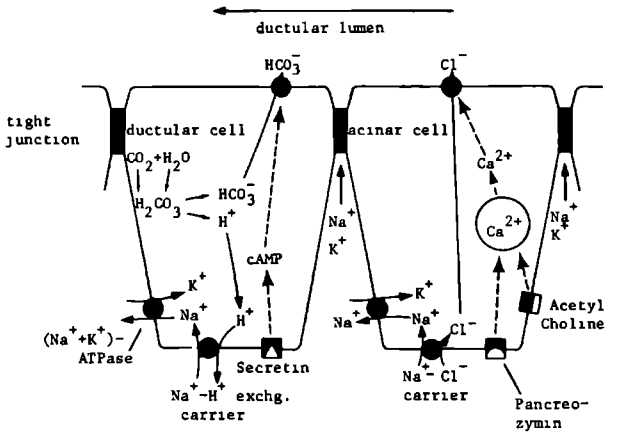


Figure 1.1

Model for fluid secretion by ductular and acinar cells of the pancreas.

1.3. Secretion of digestive enzymes

1.3.1. Intracellular aspects of the enzyme secretion process

Pancreatic fluid contains, in addition to ions, digestive enzymes or their zymogens. The same hydrolytic enzymes are present in various species, but in different proportions (see Case, 1978). The acinar cells are responsible for the enzyme secretion, for these cells contain zymogen granules, which store the enzymes untill their release.

Palade and coworkers have elucidated the intracellular route of the enzymes from synthesis to secretion by means of cell fractionation and autoradiographic techniques. They have drawn up a model for this secretory pathway (see Palade, 1975).

Fig. 1.2 shows a schematic diagram of a pancreatic acinar cell. The enzymes are synthesized on the ribosomes attached to the rough endoplasmic reticulum. This conclusion is based on the fact that shortly after pulse-labelling with ^{14}C-leucine, radioactive chymotrypsinogen is mainly found at the bound ribosomes (Siekevitz and

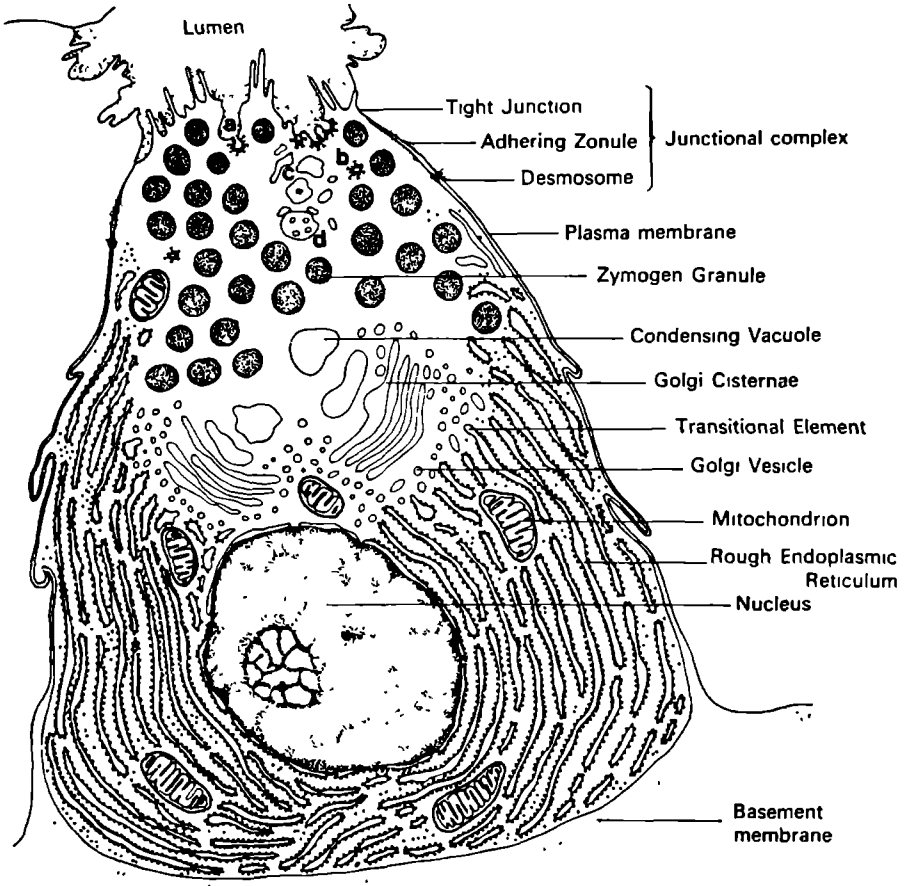


Figure 1.2

Diagram of a pancreatic acinar cell (after Case, 1978).

Palade, 1960). After synthesis the enzymes are vectorially transported into the cisternal space of the rough endoplasmic reticulum. The mechanism of this segregation process has been extensively studied by Blobel and coworkers, which has resulted in the "signal hypothesis"

(Campbell and Blobel, 1976). From the cisternal space the enzymes are transported via the small vesicles of the Golgi complex to the condensing vacuoles (Jamieson and Palade, 1967). This transport process requires energy, which can be supplied by ATP (Jamieson and Palade, 1968). The condensing vacuoles are converted into zymogen granules by concentration of the enzyme content of the vacuoles. This is a passive process, which does not require energy (Jamieson and Palade, 1971). There are indications that the proteins combine with sulfated polyanions to osmotically inactive aggregates, which causes an outflow of water into the cytoplasm (Berg and Young, 1971; Tartakoff et al., 1974). The proteins are stored in the zymogen granules, located near the apical membrane. When discharge takes place, the zymogen granules migrate to the apical membrane, possibly by interaction with the microtubular-microfilamentous system (Seybold et al., 1975; Williams and Lee, 1976). Fusion of the membrane of the zymogen granule with the plasma membrane takes place, followed by splitting and extrusion of the granule content into the lumen. Upon stimulation of the enzyme secretion there is an increase in the fusion and subsequent splitting activity, which results in an enhanced enzyme discharge. This discharge process, called exocytosis, requires energy (Jamieson and Palade, 1968, 1971).

After exocytosis excess membrane must be removed from the plasma membrane. There are indications that this occurs by an endocytotic mechanism (Kramer and Geuze, 1974). The retrieved membrane is then broken down (Geuze and Kramer, 1974) and the components are probably re-used.

Although there is much evidence for the hypothesis of vectorial transport of proteins and discharge via exocytosis, Rothman (1975) developed another theory: the equilibrium model, which is mainly based on his own observations. In this model proteins are supposed to be able to cross the membranes of the various cell organelles, which would mean that the proteins are present in all cell compartments, including the cytoplasm. The discharge of proteins would take place by means of their transport from the cytoplasm to the lumen across the plasma membrane. This model is supported by the observ-

ation that upon stimulation there is a non-parallel discharge of several digestive enzymes (Rothman, 1967; Adelson and Rothman, 1975), a phenomenon which cannot be explained by the exocytosis theory. However, in spite of this observation the segregation theory of Palade is generally accepted.

1.3.2. Regulation of enzyme secretion

The physiological stimulants of pancreatic enzyme secretion are the neurotransmitter acetylcholine and the hormone pancreozymin. Acetylcholine is released upon stimulation of the vagus nerve, while pancreozymin is liberated from the duodenum by the passage of food, as mentioned before. Because of the totally different structure of both stimulants it is obvious that there must be a separate receptor for each of them. This is confirmed by the observation that atropine blocks the effect of acetylcholine and not that of pancreozymin (Case and Clausen, 1973; Morisset and Poirier, 1977).

Pancreatic enzyme secretion can also be stimulated by gastrin, a hormone secreted by the stomach. Since the C-terminal pentapeptide of gastrin is the same as that of pancreozymin (Stening and Grossman, 1969), it can be concluded that the C-terminal part of pancreozymin is particularly important for its biological activity. This is confirmed by the finding that the synthetic C-terminal octapeptide of pancreozymin has all the biological properties of the native hormone and is even more active, on a molar basis (Ondetti et al., 1970; Robberecht et al., 1975). Recently several investigators have shown that this octapeptide, besides in the intestinal tract, also occurs in brain tissue (Muller et al., 1977; Robberecht et al., 1978b; Dockray et al., 1978). The physiological meaning of this phenomenon is not yet clear. In 1967 Anastasi et al. isolated from the skin of the Australian amphibian Hyla caerulea a decapeptide, caerulein, which resembles the C-terminal octapeptide of pancreozymin and which is a very potent stimulator of the pancreatic enzyme secretion (Dockray, 1972).

Besides the above mentioned pancreozymin-like stimulants, there are also others which do not resemble pancreozymin. Recently it has

been shown that secretin and VIP, which primarily act on the fluid secretion, also increase the enzyme secretion (Gardner and Jackson, 1977). The stimulating effect of secretin appears to be very species-dependent (De Pont et al., 1979).

Finally there is a group of stimulants whose structure is dissimilar to that of pancreozymin and secretin and whose physiological role is not yet known. Among them are bombesin, litorin and physalaemin, which can be isolated from amphibian skin, and eledoisin, which is purified from the salivary gland of a mediterranean octopod (Deschodt-Lanckman et al., 1976; May et al., 1978). Jensen and Gardner (1979) have shown that pancreatic acinar cells have specific receptors for physalaemin and eledoisin.

1.3.3. Hormone-receptor interaction

The hormone that is primarily involved in the pancreatic enzyme secretion is pancreozymin. There are some reports that peptide hormones can penetrate the intracellular space (Kolata, 1978; Bergeron et al., 1979), but Philpot and Petersen (1979) showed for pancreatic acinar cells that interaction of the stimulating hormone with the outer cell surface is necessary for cell activation. So the basal cell membrane must posses a receptor with which pancreozymin can interact. This hormone-receptor interaction triggers an intracellular process finally resulting in enzyme secretion.

So far there is not much information about the nature of the receptor for pancreozymin. Since the C-terminal octapeptide of pancreozymin has the same range of biological activities and is even more potent than pancreozymin itself (Ondetti et al., 1970; Robberecht et al., 1978), it appears that the C-terminal part of the peptide is essential for recognition and interaction with the receptor. This is confirmed by binding studies of Robberecht et al. (1978a), who found that pancreozymin, its C-terminal octapeptide, pentagastrin (Peptavlon^{R}), which has the same C-terminal tetrapeptide as pancreozymin, and caerulein, which has seven amino acids identical with the C-terminal octapeptide of pancreozymin, all act on the same receptor. Comparison of the action of these pancreozymin-like

peptides, e.g. their capacity to occupy binding sites and their biological activity can give more information about the nature of the receptor. Moreover, in recent years several derivatives of these hormones, in which one or more amino acids are replaced or modified, have become available. These derivatives are very useful for obtaining more insight in the chemical structure of the bond between hormone and receptor.

From such experiments it can be concluded that the C-terminal tetrapeptide of pancreozymin is required for binding to the receptor, which results in biological activity. Differences in potency of the several peptides, containing this C-terminal tetrapeptide, are due to differences in affinity for the receptor (Gardner et al., 1975; Robberecht et al., 1978). Gardner et al. (1975) found that replacing aspartic acid on position 2 from the C-terminal end by alanin and replacing methionine on position 3 by leucine reduces the biological response to pancreozymin-C-octapeptide. Even more important for the biological activity seems to be the sulfated tyrosine residue on position 7 from the C-terminal end. Desulfation of the peptide causes loss of affinity for the receptor and of biological activity (Gardner et al., 1975; Robberecht et al., 1978). Replacing the sulfated tyrosine by a sulfated serine reduces the potency of the peptide, which indicates that the sulfate ester by itself is not sufficient for full potency (Bodanszky et al., 1977). When the sulfated tyrosine residue is replaced by sulfated hydroxynorleucine, the resulting peptide is less active than the native peptide, but more active than the peptide with a sulfated serine on position 7. This suggests that the distance of the sulfate ester group from the peptide backbone is important for the biological activity (Bodanszky et al., 1978).

1.3.4. Stimulus-secretion coupling: cyclic AMP

The hormone-receptor interaction at the basal plasma membrane leads to alterations in the concentration of one or more intracellular components, which results in the stimulation of enzyme secretion by exocytosis. For several components there are indicat-

ions that they may be involved as a second messenger in the stimulus-secretion coupling process: cyclic AMP, calcium, sodium, cyclic GMP.

The pancreas contains an adenylate cyclase, which can be stimulated by pancreozymin (Rutten et al., 1972; Marois et al., 1972; Svoboda et al., 1976; De Pont et al., 1979). This pancreozymin-sensitive adenylate cyclase is present in the acinar cells (Kempen et al., 1977b). However, its activation does not lead to a clear increase in the cAMP level (Benz et al., 1972; Robberecht et al., 1974; Albano et al., 1976; Haymovits and Scheele, 1976) or it leads to a small increase only (Kempen et al., 1977a; Deschodt-Lanckman et al., 1975). Moreover, cholera toxin, which activates pancreatic adenylate cyclase, does not stimulate the enzyme secretion (Kempen et al., 1975; Smith and Case, 1975). Incubation of a variety of pancreatic preparations with cAMP and its analogues or with a phosphodiesterase inhibitor causes some increase in enzyme secretion, but rather high concentrations of these agents are required and the effect is small compared to that induced by pancreozymin (Kempen et al., 1977a; Benz et al., 1972; Heisler et al., 1972; Lambert et al., 1975). We must conclude that so far no convincing evidence for a role of cAMP in pancreozymin-induced enzyme secretion exists.

Secretin, which stimulates enzyme secretion in some species, also activates adenylate cyclase in these species (De Pont et al., 1979), resulting in an increased cAMP level of the pancreas (Robberecht et al., 1974, 1976). Derivatives of cAMP stimulate the enzyme secretion to about the same level as secretin (Haymovits and Scheele, 1976; Gardner and Jackson, 1977). Furthermore, the enzyme secretion is stimulated equally by secretin plus a cAMP-derivative as by secretin alone (Gardner and Jackson, 1977). These observations indicate that cAMP is involved in the secretin-induced enzyme secretion.

1.3.5. Stimulus-secretion coupling: calcium

In several tissues Ca^{2+} ions play a role as second messenger in stimulus-secretion coupling (see Douglas, 1976). Studies of the possible role of Ca^{2+} in the enzyme secretion process in the pancreas are hampered by the fact that direct measurements of the cytoplasmic

calcium concentration in the acinar cell are not yet possible. Hence, only indirect evidence for a role of calcium in the stimulus-secretion coupling is available.

The first indication for a role of calcium in pancreatic enzyme secretion was the observation that in the absence of extracellular calcium the basal and stimulated enzyme secretion are decreased (Hokin, 1966; Robberecht and Christophe, 1971; Argent et al., 1973; Case and Clausen, 1973; Petersen and Ueda, 1976; Kanno and Yamamoto, 1977). This effect occurs only after prolonged exposure to calcium-free medium, which suggests that extracellular Ca^{2+} is not necessary for the initiation of this process (Case and Clausen, 1973; Schreurs et al., 1976a; Petersen and Ueda, 1976). This is confirmed by Scheele and Haymovits (1979), who divided the stimulated discharge of enzymes into two components. One component does not depend on the extracellular calcium concentration and operates during the early period of stimulation, while the other component, operating during the entire stimulation period, depends on the extracellular calcium concentration.

Incubation in a medium, containing the calcium-ionophore A23187 in the presence of Ca^{2+} ions, stimulates enzyme secretion by various pancreatic preparations (Eimerl et al., 1974; Schreurs et al., 1976a; Poulsen and Williams, 1977). This suggests that a rise in the cytoplasmic calcium concentration is important for the stimulation of enzyme secretion. In principle, physiological stimulants can cause a rise in cytoplasmic calcium concentration in two ways: by increasing the membrane permeability for calcium resulting in a Ca^{2+} influx, or by releasing calcium from an intracellular pool. The first possibility is supported by the observation of Kondo and Schulz (1976b) and of Heisler and Grondin (1973) that stimulation of the enzyme secretion causes an increased uptake of $^{45}Ca^{2+}$. However, when pancreatic fragments or acinar cells are preloaded with $^{45}Ca^{2+}$, addition of a stimulus causes an efflux of $^{45}Ca^{2+}$ (Case and Clausen, 1973; Schreurs et al., 1975; Gardner et al., 1975). This suggests that upon stimulation of the enzyme secretion there is a release of calcium from an intracellular pool. Another indication for such a release is

presented by Chandler and Williams (1978a, b). They load pancreatic acini with chlorotetracycline, a fluorescent probe which easily incorporates into membranes, the fluorescence of which is sensitive for calcium. They find that the chlorotetracycline fluorescence decreases upon addition of a stimulant.

These observations suggest that a rise in the cytoplasmic calcium concentration is an important step in stimulus-secretion coupling, but it is not yet clear how this leads to exocytosis. Ca^{2+} ions may be directly involved in the fusion of the zymogen granule membrane with the plasma membrane by reducing the electrostatic repulsion between the two negatively charged membranes (Dean, 1975). However, this effect is not specific for Ca^{2+}, but can be produced by other divalent cations like Mg^{2+}. Since Mg^{2+} is present in high concentration in the cytoplasm and does not seem to play an important role in stimulus-secretion coupling (Schreurs et al., 1976b), this explanation for the role of calcium is unlikely. On the other hand, there appears to be an absolute requirement of Ca^{2+} for the fusion between insulin granules isolated from mouse β cells and plasma membranes from cod islets (Davis and Lazarus, 1976).

Another possibility is that Ca^{2+} acts on the microtubular system, which may be involved in the migration of the granules to the plasma membrane (Gaskin et al., 1975; Schliwa et al., 1976). However, this is a rather slow effect compared to exocytosis. Baker (1977) has developed an interesting hypothesis for the role of calcium in the control of neurosecretion. He suggested that the cytoplasm, which is a rather viscous gel under basal conditions, may be liquified under the influence of calcium. This gel-sol conversion might facilitate the motion of granules to the plasma membrane.

Summarizing it can be concluded that a rise in the cytoplasmic calcium concentration is an important step in the enzyme secretion process, but it is still unknown how this increase in the calcium concentration results in stimulation of exocytosis.

1.3.6. Stimulus-secretion coupling: sodium

There are several indications that sodium ions are involved in

pancreatic enzyme secretion. Lowering the sodium concentration of the incubation medium inhibits stimulation of the enzyme secretion in various pancreatic preparations (Case and Clausen, 1973; Williams et al., 1976; Petersen and Ueda, 1976; Kanno et al., 1977).

Further evidence comes from electrophysiological experiments. Under normal conditions stimulation of pancreatic enzyme secretion by acetylcholine or pancreozymin causes depolarization of the acinar cell membrane (Matthews and Petersen, 1973; Matthews et al., 1973; Greenwell, 1975; Petersen and Ueda, 1975; Poulsen and Williams, 1977). The extent of the depolarization depends on the extracellular sodium concentration (Matthews and Petersen, 1973; Nishiyama and Petersen, 1975), which suggests that the depolarization is due to an increase in the membrane permeability for sodium and a Na^+ influx by the stimulant. In agreement with this, Case et al. (1978) have measured an increased Na^+ influx in rat pancreatic fragments under the influence of acetylcholine or caerulein. Mainly on the basis of electrophysiological experiments, Petersen and Ueda (1976) assume that the membrane permeability for sodium is regulated by a release of Ca^{2+} from the plasma membrane upon stimulation.

There thus appears to be considerable evidence that Na^+ ions are involved in enzyme secretion. However, it is not yet known whether the increased uptake of sodium upon stimulation is an important step in stimulus-secretion coupling or whether it is only a secondary effect of stimulation.

1.3.7. Stimulus-secretion coupling: cyclic GMP

Hormonal or cholinergic stimulation of enzyme secretion causes an increase in the cGMP level in a variety of pancreatic preparations (Robberecht et al., 1974; Albano et al., 1976; Haymovits and Scheele, 1976; Christophe et al., 1976b; Kapoor and Krishna, 1977; Lopatin and Gardner, 1978). This increase is probably not the result of direct activation of guanylate cyclase by the stimulant, but is due to the rise in the cytoplasmic calcium concentration (Christophe et al., 1976b). The role of this rise in cGMP content is not yet understood. Exogenous cGMP or its derivatives do not stimulate pancreatic enzyme

secretion (Lambert et al., 1975; Haymovits and Scheele, 1976). Recently it has been shown that some agents stimulate enzyme secretion without elevating the cGMP content of the pancreas, and that a rise in cGMP level not always results in enhanced enzyme release (Gunther and Jamieson, 1979; Gardner and Rottman, 1980). These observations suggest that an important role of cGMP in pancreatic enzyme secretion is unlikely.

1.4. Aims of our study

The purpose of our investigation is to obtain more insight in the regulation of the pancreatic enzyme secretion on the cellular level. We have directed our attention mainly to two points: the hormone-receptor interaction and the role of possible second messengers in the stimulus-secretion coupling.

As discussed in section 1.3.3, it is clear that the C-terminal octapeptide of the hormone pancreozymin is essential for the hormone-receptor interaction. It has been shown that the tryptophan residue in the C-terminal part of gastrin and pancreozymin is essential for their biological activity (Morley, 1976; Yabe et al., 1977). We have investigated which property of tryptophan is essential for the interaction between hormone and receptor, using derivatives of pancreozymin-C-octapeptide in which the tryptophan residue is modified in several ways.

At the beginning of our study there was already strong evidence that a rise in the cytoplasmic calcium concentration is an important step in the stimulus-secretion coupling (Eimerl et al., 1974; Schreurs et al., 1976a; Poulsen and Williams, 1977). However, there was still disagreement whether this rise is caused by the release of calcium from an intracellular pool (Gardner et al., 1975) or by an increase in membrane permeability for calcium (Kondo and Schulz, 1976a, 1976b). To obtain more insight in this question, the calcium metabolism in the pancreatic acinar cell has been studied.

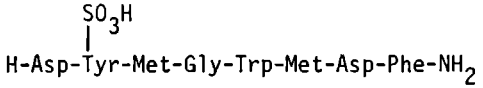
Since there are indications that - in addition to calcium - cyclic AMP and Na^+ ions are also involved as second messengers in

the stimulus-secretion coupling, their role in the pancreatic secretion, especially in relation to the behavior of calcium ions, has been studied.

CHAPTER II.

2.1. Introduction

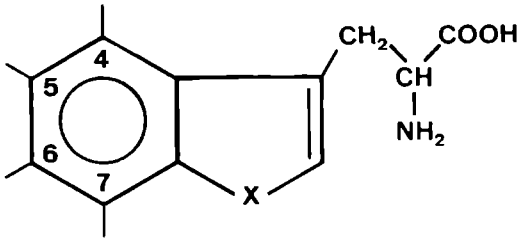
Pancreatic enzyme secretion is stimulated by the hormone pancreozymin, which contains 33 amino acids (Mutt and Jorpes, 1968). Its C-terminal octapeptide (Trp-P8) exhibits the whole range of biological activities of the complete hormone (Anastasi et al., 1968; Ondetti et al., 1970; Gardner et al., 1975). Its structure is as follows:



The C-terminal pentapeptide of pancreozymin (and thus of Trp-P8) is identical with that of gastrin and has the full activity of the latter hormone (Tracey and Gregory, 1964; Morley et al., 1965). The pentapeptide stimulates pancreatic enzyme secretion only in much higher concentrations, probably because of the lack of the sulfate ester group of the tyrosyl-O-sulfate ester residue (Bodanszky et al., 1977, 1978; Yajima et al., 1976).

The tryptophan residue in the C-terminal part of both gastrin and pancreozymin is essential for the biological function of both peptides (Morley et al., 1965; Morley, 1968; Yabe et al., 1977). In order to study this functional role of the tryptophan residue, six derivatives of L-tryptophan are synthesized (Rajh et al., 1979), which closely resemble the structure of tryptophan, but differ in hydrogen bonding, charge-transfer capacity and hydrophobicity (Fig. 2.1). With these tryptophan derivatives six analogues of the C-terminal octapeptide have been prepared (Rajh et al., 1980). The synthesis of the octapeptide has been carried out in such a way that the C-terminal tetrapeptides are obtained as intermediates.

In this study we have tested the octapeptide analogues, and partially also the tetrapeptide analogues, on rat pancreas adenylate cyclase activity, on amylase secretion by rabbit pancreas fragments



Modification	Name	Abbreviation
X=O	Benzofurylalanine	Bfa
X=S	Benzothienylalanine	Bta
X=N(CH_3)	N^{in}-methyl-tryptophan	Trp(Me)
X=NH, 5-monofluoro	5-Fluorotryptophan	Trp(5F)
X=NH, 6-monofluoro	6-Fluorotryptophan	Trp(6F)
X=NH, 4,5,6,7-tetrafluoro	4,5,6,7-Tetrafluorotryptophan	Trp(tF)
X=NH	Tryptophan	Trp

Figure 2.1.

Tryptophan derivatives and their abbreviations.

and on the in vivo secretion of the rat pancreas. The experiments have been carried out in collaboration with Dr. H.M.Rajh, Dr. M.J. Smyth and Dr. J.W.C.M.Jansen.

2.2. Materials and methods

2.2.1. Materials

[α-^{32}P]ATP (5-10 Ci/mmol) and cyclic [8-^{3}H]AMP are obtained from the Radiochemical Centre, Amersham, U.K. The Phadebas amylase test is purchased from Pharmacia, Uppsala, Sweden.

2.2.2. Pancreozymin analogues

The hormone analogues are synthesized by Dr. H.M.Rajh in collab-

oration with Dr. G.I.Tesser and Prof.Dr. R.J.F. Nivard, (Department of Organic Chemistry, University of Nijmegen, Nijmegen, The Netherlands) and stored in vacuo at -20 ^{o}C. The octapeptides are freshly dissolved in water, whereas the tetrapeptides are dissolved in 1% dimethyl sulfoxide. Control experiments have shown that dimethyl sulfoxide in the maximal final concentration used has no effect on the adenylate cyclase activity and the secretory activity of the rat pancreas in vivo.

2.2.3. Adenylate cyclase assay

The adenylate cyclase assay is carried out with an 8000x g pellet of a rat pancreas homogenate, obtained as previously described (De Pont et al., 1979). The method of Rutten et al. (1972) is used for the enzyme assay with two exceptions: 1. whenever peptides are present, 1 mg/ml phosphatidylserine is added to the incubation medium in order to protect the hormone receptors (Kempen et al., 1974), 2. cyclic AMP is separated from ATP according to Salomon et al. (1974) by means of two successive column chromatographic procedures on Dowex 50 W-X4 cation-exchange resin and neutral alumina. The recovery of the chromatographic procedure is determined by adding 3000 cpm cyclic [8-^{3}H]AMP to each tube before centrifugation. Protein is determined in an aliquot of the enzyme suspension according to Lowry et al. (1951), using bovine serum albumin as a standard.

2.2.4. Enzyme secretion by pancreas fragments

Fragments of 50-100 mg wet weight are cut from freshly isolated rabbit pancreas and are incubated in 1 ml Krebs-Ringer bicarbonate medium. The composition of this medium is: NaCl,118,5 mM; KCl,3.5 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 1.2 mM and glucose, 5.8 mM, pH 7.4. After 30 min the medium is replaced and the experiment is started. After 0, 20 and 40 min 100 µl samples of the medium are taken. The peptides are added after 20 min. After 40 min the fragment is homogenized in a Potter tube in the residual 700 µl incubation medium. Amylase activity is measured in appropriate dilutions of the homogenate and the medium samples by means of the

Phadebas test, modified for assay on a microscale. The release of amylase caused by the addition of the peptides is expressed as percentage of the original amylase content of the fragments and compared with the release caused by 10^{-7}M Trp-P8 in a parallel experiment.

2.2.5. Pancreatic secretion in vivo

The experiments with the rat pancreas in vivo are carried out as previously described (Kempen et al., 1975). In principle, the pancreatic duct is cannulated, the bile diverted and the pylorus ligated. An infusion cannula is introduced into a femoral vein, allowing administration of peptides from a syringe. The peptides are given as 100 µl bolus injections after which immediately 100-150 µl 0.9% NaCl solution is injected through the same cannula. Each fraction of pancreatic fluid is drawn from the tygon collecting tubing (0.28 mm internal, 0.61 mm external diameter) into a precision microsyringe (10 µl maximal volume). The fraction volume is determined by reading the distance between the two menisci. The sample is delivered to a 10-ml plastic tube and diluted with 4.0 ml water. The protein concentration in the diluted samples is estimated by determining the 280 nm absorbance, with bovine serum albumin serving as standard.

2.3. Results

2.3.1. Rat pancreas adenylate cyclase

The adenylate cyclase activity of the rat pancreas is stimulated by the C-terminal octapeptide of pancreozymin (Kempen et al., 1974; Svoboda et al., 1978). Fig.2.2 shows that the maximal activity is reached at an octapeptide concentration of 3.10^{-7}M, whereas half-maximal activity is obtained at 4.10^{-8}M. The two monofluorinated octapeptides Trp(5F)-P8 and Trp(6F)-P8 present the same behaviour. Fig.2.2 also shows that the four other peptides have a 30-100 fold lower affinity and that the maximal response reached is only 50-75% of that of the unmodified octapeptide, even at concentrations of

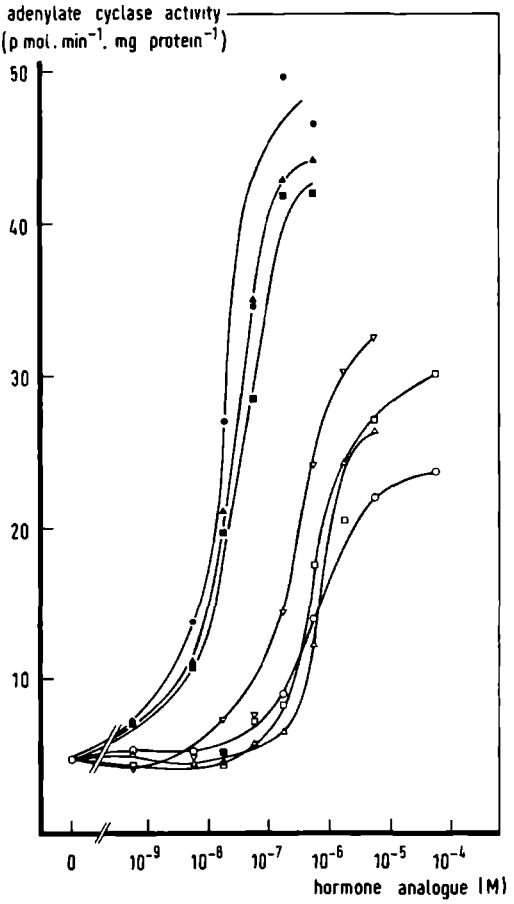


Figure 2.2.

Effect of analogues of the C-terminal octapeptide of pancreozymin on the adenylate cyclase activity of a rat pancreas perticulate fraction. The following symbols are used: -▲- Trp-P8; -■- Trp(5F)-P8; -●- Trp(6F)-P8; -▽- Trp(Me)-P8; -Δ- Trp(tF)-P8; -□- Bta-P8; -○- Bfa-P8. Values represent averages of 4-6 experiments.

Table 2.1. EFFECTS OF ANALOGUES OF PANCREOZYMIN-C-OCTAPEPTIDE ON RAT PANCREAS ADENYLATE CYCLASE ACTIVITY IN THE PRESENCE OR ABSENCE OF DIBUTYRYL-cGMP

Hormone analogue added (10^{-6}M)	Adenylate cyclase activity (pmoles.min^{-1}.mg protein^{-1})	
	no dbcGMP	1 mM dbcGMP
-	3.6 ± 0.3 (38)	4.6 ± 0.6 (4)
Trp-P8	48 ± 3.0 (20)	16.3 ± 0.3 (5)
Trp(5F)-P8	43 ± 3.3 (12)	13.9 ± 0.8 (3)
Trp(6F)-P8	47 ± 4.3 (12)	20 ± 3.3 (3)
Trp(Me)-P8	23 ± 1.6 (13)	3.6 ± 0.5 (4)
Trp(tF)-P8	16 ± 2.0 (12)	4.5 ± 0.4 (3)
Bta-P8	17 ± 2.0 (15)	3.7 ± 0.8 (3)
Bfa-P8	14 ± 1.6 (15)	4.0 ± 0.3 (3)
NaF, 10^{-2}M	55 ± 3.7 (37)	

Values represent means with SEM and between parentheses the number of experiments.

10^{-4} to 10^{-5}M. Table 2.1 shows the adenylate cyclase activities in the presence of the seven octapeptides at 10^{-6}M concentration for a large number of experiments. The activity with Trp(Me)-P8 is slightly, but significantly, higher than with Trp(tF)-P8, Bta-P8 and Bfa-P8, which show approximately the same activities at this concentration.

In order to determine whether the maximal obtainable enzyme activity with one of the latter octapeptides is indeed lower than that obtained with Trp-P8, the dose-response curve for Bfa-P8 has been measured in the presence and absence of 10^{-7}M Trp-P8. Fig.2.3 shows that at Bfa-P8 concentrations below 10^{-6}M the adenylate cyclase activity is determined by the Trp-P8 present. At 10^{-5}M Bfa-P8 the Trp-P8 begins to lose its effect and at 10^{-4}M Bfa-P8 it has completely lost its effect. This suggests that Bfa-P8 and Trp-P8 compete for

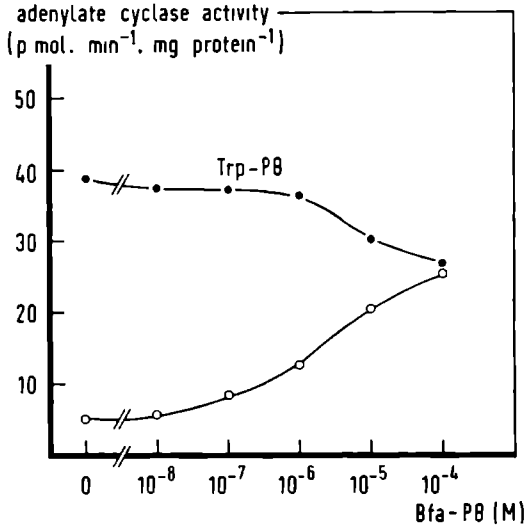


Figure 2.3.

Effects of varying concentrations of Bfa-P8 on the adenylate cyclase activity of a rat pancreas particulate fraction measured in the absence (-0-) or presence (-●-) of 10^{-7}M Trp-P8. Values are means of two experiments with duplicate measurements.

the same receptor site of the adenylate cyclase system, with the unmodified octapeptide having a 100-times higher affinity. At concentrations where the receptor site is occupied by one of the two peptides, the activity is determined by the concentration of the occupying substance. Replacement of the nitrogen atom in the indolyl residue by an oxygen atom apparently lowers the activity of the octapeptide towards the adenylate cyclase.

Peikin et al. (1979) have recently found that dibutyryl cyclic GMP (dbcGMP) inhibits the effects of pancreozymin and its analogues on the enzyme secretion and also on the calcium efflux from isolated guinea pig acini. Petersen and Philpott (1979) report that the effect of the pancreozymin analogue caerulein on the membrane potential and

resistance in the mouse pancreas is also inhibited by this nucleotide. Robberecht et al. (1979) have shown that dbcGMP inhibits the amylase release from rat pancreatic fragments by pancreozymin, the binding of the hormone and the adenylate cyclase activation by the hormone in rat pancreatic plasma membranes. These findings indicate that dbcGMP is a competitive antagonist of pancreozymin in a number of biological functions. In Table 2.1 we show the effect of 1 mM dbcGMP on the stimulation of rat pancreatic adenylate cyclase by the seven octapeptides in 10^{-6}M concentration. The stimulatory effect of the four peptides with low activity is completely abolished and the activity of Trp-P8 and the two monofluorinated peptides is inhibited by ca 70%. Complete inhibition of the effects of the latter three octapeptides by 1 mM dbcGMP is obtained, when these are applied in 10^{-7}M concentration (not shown). This indicates that the competitive effect of dbcGMP also takes place with the peptides with a modified tryptophan residue.

The effects of the tetrapeptides on the adenylate cyclase activity have also been tested (Table 2.2). In 10^{-4}M concentration tetragastrin and its two monofluorinated analogues stimulate the adenylate cyclase activity to slightly more than twice the basal activity. Trp(Me)-P4, Bfa-P4 and Bta-P4 do not stimulate the adenylate cyclase activity. The tetrafluorinated tetrapeptide (Trp(tF)-P4) is quite active, its effect being twice that of the unmodified tetrapeptide. Since the large effect of the tetrafluorinated compound might be due to its hydrophobicity, we have also tested a derivative of Trp-P4 in which the NH_2 group of the tryptophan residue is blocked by a Boc group (Boc-Trp-P4). The effect of the latter compound is also nearly twice as high as that of free tetragastrin (Table 2.2).

2.3.2. Rabbit pancreatic fragments

The C-terminal octapeptide stimulates amylase release from rabbit pancreas fragments. The maximal effect is obtained with 10^{-8}M Trp-P8, whereas 50% stimulation is obtained at 2.10^{-9}M (Fig.2.4). The two monofluorinated analogues of the octapeptide have the same activity as the unmodified octapeptide. The dose-response curves of the four

Table 2.2. EFFECTS OF ANALOGUES OF THE C-TERMINAL TETRAPEPTIDE OF PANCREOZYMIN ON RAT PANCREAS ADENYLATE CYCLASE ACTIVITY

Hormone analogue added (10^{-4}M)	Adenylate cyclase activity (pmol.min^{-1}.mg protein^{-1})
-	4.4 ± 0.4 (6)
Trp-P4	9 ± 1.2 (6)
Trp(5F)-P4	10 ± 1.4 (5)
Trp(6F)-P4	11 ± 1.3 (5)
Trp(Me)-P4	5.4 ± 0.3 (6)
Trp(tF)-P4	20 ± 2.6 (6)
Bta-P4	5.0 ± 0.3 (5)
Bfa-P4	4.9 ± 0.5 (5)
Boc-Trp-P4	16 ± 2.4 (4)
NaF	55 ± 3.7 (37)

Values represent means with S.E.M. and between parentheses the number of experiments.

other analogues are shifted 1.5-2 log units to the right, Trp(Me)-P8 having the highest activity and Trp(tF)-P8, Bta-P8 and Bfa-P8 having slightly lower and equal activities. At the highest concentration used in these experiments (10^{-6}M) the effects of the latter four octapeptides are still less than that obtained with a maximally stimulating concentration of the unmodified octapeptide.

2.3.3. Rat pancreas in vivo

The rat pancreas in vivo has a low rate of fluid and protein secretion. Upon injection of pancreozymin or its C-terminal octapeptide both parameters are increased, in such a way that the protein concentration in the secreted fluid also increases. Fig. 2.5 shows an experiment in which all seven octapeptides are given in 75 ng amounts at 50 min intervals. It is clear that the two monofluorinated

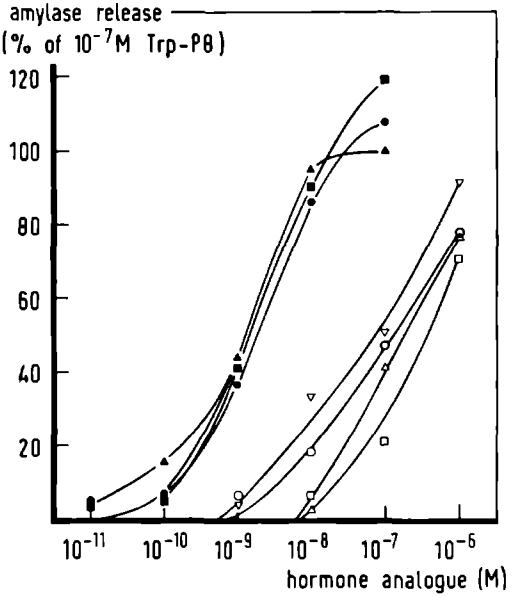


Figure 2.4.

Effects of analogues of the C-terminal octapeptide of pancreozymin on the amylase release by rabbit pancreas fragments. Effects are expressed as percent of the effect of 10^{-7}M Trp-P8 measured in the same experiment. Symbols as in Fig.2.2. The basal release in the 20-min experimental period is 1.3 ± 0.13 (n=41) % of the amylase present in the slices. With 10^{-7}M Trp-P8 the release is 7.8 ± 0.63 (n= 38) %. Values represent averages of 3-5 experiments.

analogues (Trp(5F)-P8 and Trp(6F)-P8) elicit approximately the same response as the unmodified octapeptide (Trp-P8), while the response to the other four analogues is much smaller. In order to obtain a better comparison of these four analogues, they have been injected in 150 ng amounts and compared with the 15 ng of the unmodified octapeptide (Fig.2.6). The response to the N-methylated analogue (Trp-(Me)-P8) is still somewhat less than that to the ten-fold lower dose

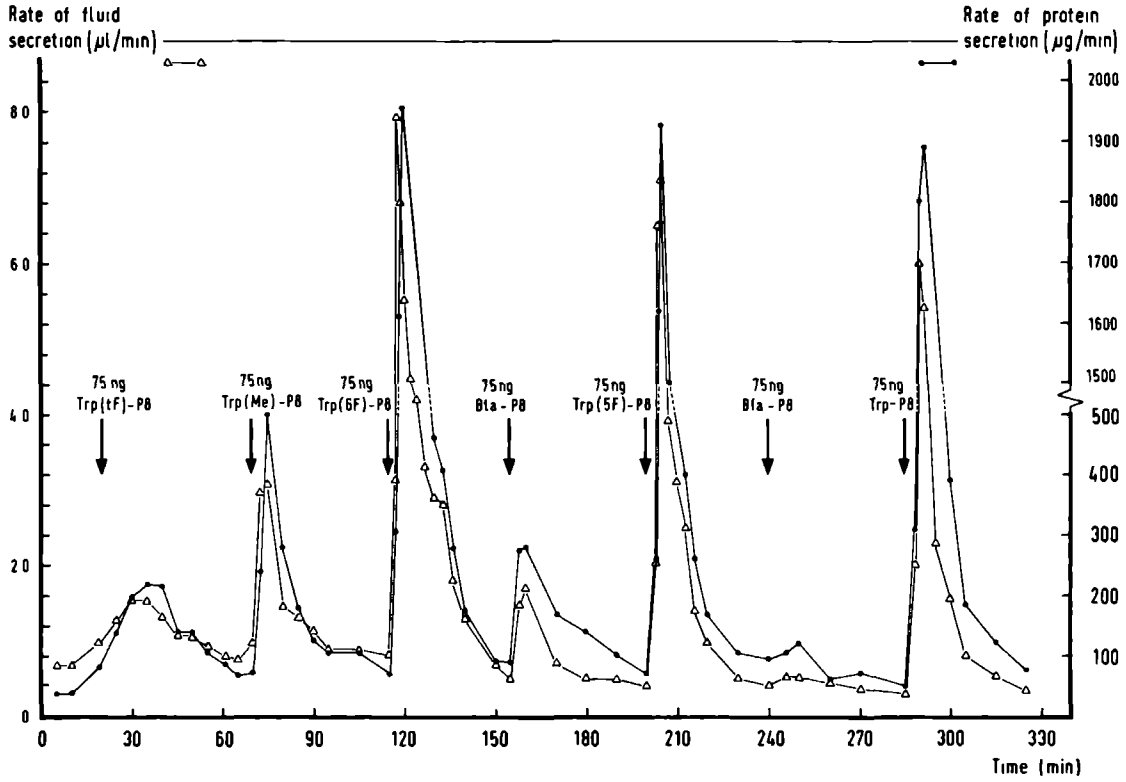


Figure 2.5.

Effects of analogues of the C-terminal octapeptide of pancreozymin on the rates of fluid (-Δ-) and protein (-●-) secretion by the rat pancreas in vivo. Injections of 75 ng were given at the indicated times (↓).

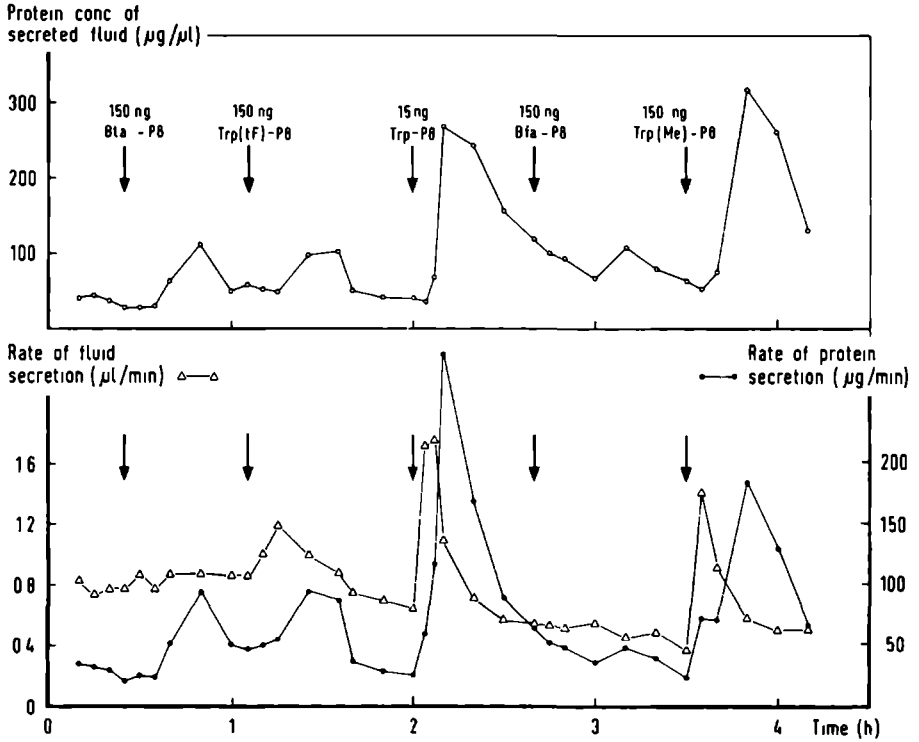


Figure 2.6.

Effects of analogues of the C-terminal octapeptide of pancreozymin on the rates of fluid (-Δ-) and protein (-●-) secretion by the rat pancreas in vivo. In the upper part of this figure the protein concentration of the secreted fluid (-o-) is given. Injections of the analogues (15 ng of Trp-P8 and 150 ng of the other peptides) are given at the indicated times (↓).

of the unmodified octapeptide. The response to the three other octapeptides is very low, in particular those to Bfa-P8.

In these experiments the peak of the protein secretion generally appears later than that of the fluid secretion. This is due to the fact that an increase in fluid secretion in the acinar region imme-

diately results in an increased appearance of fluid at the end of the duct, whereas the additionally secreted protein must first be transported along the entire ductular system. At high rates of fluid secretion (Fig.2.5) the time difference becomes very small. As already mentioned, the relative increase of the protein secretion is larger than that of the fluid secretion, e.g. the maximal increase in fluid secretion upon addition of 15 ng Trp-P8 is 2.7-fold, whereas there is an 11.7-fold increase in protein secretion (Fig.2.6). This probably explains why analogues which stimulate the enzyme secretion only weakly (Bta-P8 and Bfa-P8) do not cause a detectable increase in fluid secretion (Fig.2.6).

In order to obtain adequate responses with the tetrapeptides, approximately 1000 times higher concentrations than for the octapeptides are necessary. Even then they have very little effect on the fluid secretion. The effect of the two monofluorinated tetrapeptides (50 µg) on the enzyme secretion is approximately equal to that of the unmodified tetrapeptide. Injections of 50 µg of Trp(Me)-P4, Bta-P4 and Bfa-P4 does not result in any response (not shown). The tetrafluorinated tetrapeptide (Trp(tF)-P4) shows a relatively large effect, even larger than that of the unmodified tetrapeptide (Trp-P4), on the fluid secretion as well as on the protein output (Fig.2.7).

In some experiments tetrapeptides, in which the N-terminal amino group of the tryptophan residue or its derivative is blocked by either a Msc group (in the case of Trp(Me)-P4) or a Boc group (all other tetrapeptides) have been used. These blocked tetrapeptides are generally more effective than the corresponding free tetrapeptides. They stimulate the fluid secretion in parallel with the enzyme secretion. The order of the potency of the blocked analogues is the same as that of the free tetrapeptides.

2.4. Discussion

The tryptophan residue on place 30 of the hormone pancreozymin seems to be very important for the effects of this hormone on the

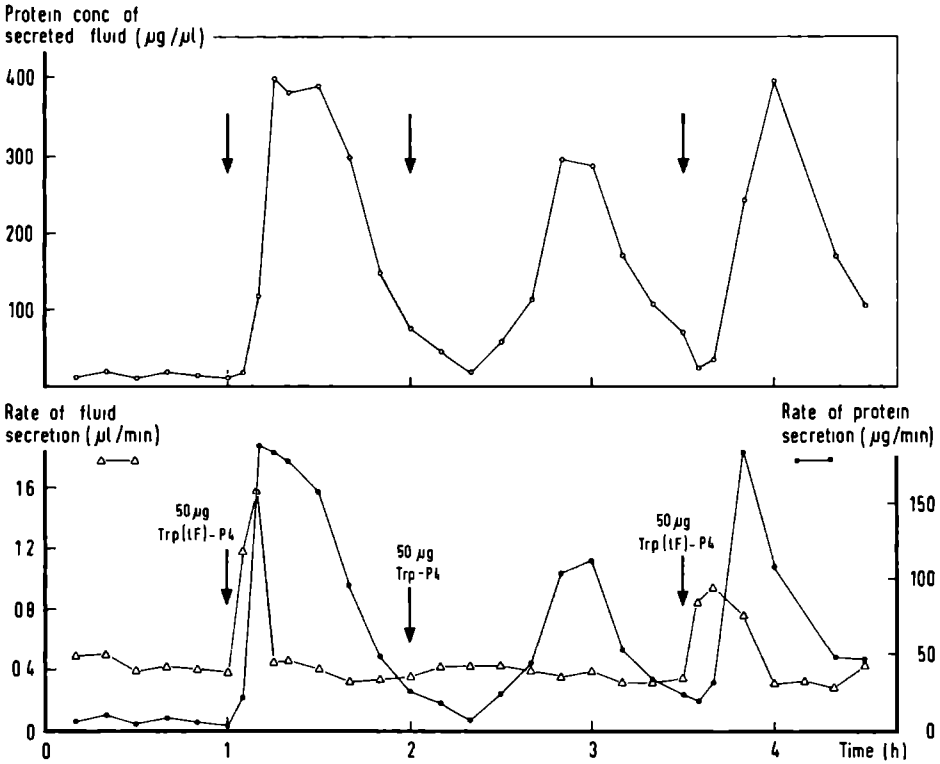


Figure 2.7.

Effects of Trp-P4 and Trp(tF)-P4 on the rates of fluid (-Δ-) and protein (-●-) secretion by the rat pancreas in vivo. In the upper part of this figure the protein concentration in the secreted fluid (-O-) is given. Injections of 50 µg of the peptides are given at the indicated times (↓).

exocrine pancreas. Slight modifications of the indolyl ring of the tryptophan residue, which have little effect on the gross structure of the amino acid, have large effects on the biological activity of the C-terminal octapeptide of the hormone. This has been determined by observing the effects on the adenylate cyclase activity in a rat

pancreas particulate fraction, the release of amylase from rabbit pancreas fragments and the protein and fluid secretion in the rat pancreas in vivo.

Methylation of the nitrogen atom in the indolyl ring of tryptophan leads to a 30-fold lowering of the potency of the octapeptide in stimulating the adenylate cyclase system as well as the release of amylase from rabbit pancreas fragments. The maximal response of the octapeptide for the enzyme is reduced by 25%. The stimulatory effect of the N-methylated octapeptide on the rat pancreas in vivo is at least 10 times lower than that of the unmodified octapeptide. Since methylation of the nitrogen atom in the indolyl ring prohibits the formation of a hydrogen bond via this atom, this suggests that hydrogen bridge formation via this nitrogen atom plays a role either in the binding to or in the activation of the pancreozymin receptor. Alternatively, the rather bulky methyl group may prevent the interaction of the N-methylated analogue with the receptor.

The three fluorinated analogues were developed to modify the ability of the indolyl ring to donate electrons to an electron acceptor in the receptor system (charge-transfer property). Actually, the charge-transfer capacity of the two monofluorinated derivatives of tryptophan is not less than that of tryptophan (Rajh et al., 1979), in contrast to a report by Coy et al. (1974). Since the structural difference with tryptophan is also very minor (one fluorine atom instead of a hydrogen atom), it is not surprising that the properties of the two monofluorinated octapeptides are indistinguishable from those of the unmodified octapeptide. The tetrafluorinated derivative of tryptophan, however, has a very low charge-transfer capacity (Rajh et al., 1979). In all three test systems the affinity and activity of the tetrafluorinated octapeptide are even lower than those of the N-methylated analogue. This strongly suggests that the charge-transfer capacity of the tryptophan residue is of importance for the biological activity of pancreozymin.

Replacement of the nitrogen atom in the indolyl ring of tryptophan by either an oxygen or a sulfur atom precludes hydrogen bond formation with a hydrogen accepting hetero atom. In addition, it

greatly lowers the charge-transfer capacity (Rajh et al., 1979). In all three test systems the octapeptide with either an oxygen or a sulfur atom in the indolyl ring shows approximately the same low activity and affinity as the tetrafluorinated analogues. This confirms that the charge-transfer capacity is important for the mechanism of action of pancreozymin. The fact that the additional lack of hydrogen bridge formation in these two analogues does not further reduce the affinity or activity, as compared to the tetrafluorinated analogue, may suggest that hydrogen bond formation is not very important for the action of pancreozymin and that the reduced activity of the N-methylated analogue is mainly due to steric hindrance of the methyl group. This conclusion is a tentative one, which requires further experimental support.

The C-terminal tetrapeptide of pancreozymin (tetragastrin) is a weak agonist on the pancreatic secretion in vivo and on the adenylate cyclase activity. The effects of the tetrapeptide analogues approximately parallel those of the octapeptides, with one interesting exception. Tetrafluorination of the tryptophan residue in the octapeptide leads to a 100-fold decrease in affinity in the three test systems, whereas the tetrafluorinated tetrapeptide stimulates the adenylate cyclase and the secretion of the rat pancreas in vivo much better than the unmodified tetrapeptide. A possible explanation for this anomalous behaviour is offered in the next paragraph.

The affinity of the unmodified tetrapeptide is approximately 1000 times lower than that of the corresponding octapeptide. This difference is most likely due to the lack of the sulfated tyrosine residue in the tetrapeptide (Bodanszky et al., 1977, 1978; Yajima et al., 1976). For the tetrafluorinated tetrapeptide the affinity for the adenylate cyclase is only a factor 10 less than for the corresponding octapeptide. An explanation might be that the tetrafluorination makes the peptide more hydrophobic, which could enhance its binding to the receptor. In the octapeptide the presence of the sulfate ester may overcome the effect of the increased hydrophobicity. This hypothesis is supported by our finding that the tetrapeptide with a blocked amino group, which is expected to be more hydrophobic

than the free tetrapeptide, is a better stimulant for the adenylate cyclase system and the rat pancreas in vivo.

The parallelism between the effects of the various analogues on the three parameters measured in this study suggests that one type of pancreozymin receptor is involved in all three systems. The peptide concentrations needed to stimulate the rat pancreas adenylate cyclase activity are, however, at least tenfold higher than for the amylase release from rabbit pancreas fragments. This difference in affinity has generally been observed (Robberecht et al., 1978a; Long and Gardner, 1977). Two explanations for this observation, between which they cannot distinguish, have been proposed by Robberecht et al. (1978a). These authors compared the effects of a series of pancreozymin analogues on [^{3}H]caerulein binding, adenylate cyclase activity, ^{45}Ca efflux and amylase release in various rat pancreas preparations. On the basis of their results, they consider 1. the presence of spare receptors, which would not be needed for maximal secretion, and 2. the existence of high- and low-affinity receptors linked to two different effector systems, such that the relative affinities of these two classes of receptors for pancreozymin analogues would be equal. Long and Gardner (1977), on the other hand, have found quantitative differences between the effects of certain analogues on ^{45}Ca efflux and adenylate cyclase activity, and thus they conclude that different receptors are involved in the adenylate cyclase activation and for the other parameters (^{45}Ca efflux, cGMP increase and enzyme release). Inasfar as the tryptophan residue in pancreozymin is involved, our results do not support the two-receptor theory, but rather support the spare receptor concept. For maximal stimulation of the adenylate cyclase activity the receptors would have to be more completely occupied than for stimulation of the enzyme secretion.

Tryptophan residues also play an important role in the action of other hormones. However, modification of these residues leads to rather variable results in the different hormones. Replacement of tryptophan by pentamethyl-L-phenylalanine, which keeps the charge-transfer capacity intact, leads to a complete loss of biological

activity in ACTH, β-corticotrophin (1-24) and tetragastrin (Van Nispen, 1974; Van Nispen et al., 1977a, 1977b). In α-MSH the same substitution leads to a decrease of the lipolytic activity, whereas the melanocyte-stimulating effect is preserved (Van Nispen et al., 1977a, 1977b). In LH-RH this substitution results in a product which has 70% of the activity of the natural compound (Coy et al., 1974). Replacement of tryptophan by 3-(1-naphtyl)-L-alanine in tetragastrin leads to a nearly complete loss of its activity on gastric secretion, whereas in LH-RH the activity is increased 1.9-fold (Yabe et al., 1976, 1977). In somatostatin monofluorination at place 5 of tryptophan leads to a 4.7-fold increase in activity (Meyers et al., 1978), whereas in LH-RH the activity is only 6% of that of the natural analogue (Coy et al., 1974).

These observations indicate that the role of tryptophan is probably different in each of the hormones tested and that the charge-transfer capacity of the indolyl ring is not the unifying basic principle for the action of tryptophan in all these hormones. In pancreozymin, however, this property of tryptophan seems to be of primary importance for its action on the pancreas, while hydrogen bond formation and hydrophobicity of the indolyl ring may play a secondary role.

CHAPTER III.

INTERACTION OF LIPOSOMES WITH ISOLATED ACINAR CELLS

3.1. Introduction

As we have mentioned before, all evidence that a rise in the cytoplasmic calcium concentration is a crucial step in the pancreatic stimulus-secretion coupling is based on indirect evidence.

In recent years a technique has been developed to bring compounds into a cell through the use of small unilamellar phospholipid vesicles, so-called liposomes (see Poste et al., 1976; Tyrell et al., 1976). Liposomes have been used to introduce biologically active compounds, like drugs or enzymes, into cells in vivo (see Tyrell et al., 1976; Pagano and Weinstein, 1978). It appears that liposomes are also a suitable tool to introduce several compounds into cells in vitro or in culture. Papahadjopoulos et al. (1974) have demonstrated that incubation of mouse 3T3 cells with liposomes containing cyclic AMP results in a significant uptake of the nucleotide by the cells. Theoharides and Douglas (1978) have found that histamine secretion by mast cells, in which process a rise in the cytoplasmic calcium concentration is supposed to play a role, can be stimulated by incubating the cells together with calcium-containing liposomes. Incubation of adrenal glands in the presence of liposomes containing calcium or sodium also results in an increased catecholamine release (Gutman et al., 1979).

Thus it seems possible to inject all kinds of substances, also those which normally do not cross the plasma membrane, into a cell by means of liposomes. Hence, liposomes may be a very useful tool to study the role of several compounds as second messenger in the pancreatic stimulus-secretion coupling. It would not only be possible to raise the cytoplasmic calcium concentration of isolated acinar cells by incubating them in the presence of calcium-containing liposomes, but also to insert into the cell a calcium-sensitive

dye like arsenazo III, which will then allow to measure changes in the cytoplasmic calcium concentration (Dunham et al., 1977). Another possibility would be to introduce other putative second messengers, like cyclic AMP or cyclic GMP, into the cell and to observe their effects on enzyme release.

So we have prepared liposomes of various compositions, incubated them together with isolated acinar cells and checked whether the liposome content is transferred into the cytoplasm.

3.2. Materials and methods

3.2.1. Materials

Cholesterol and dimyristoyl phosphatidylcholine are purchased from Sigma Chemical Co. (St.Louis, Mo, USA), stearylamine and octadecylamine from Koch-Light Lab. (Colnbrook, England) and 6-carboxyfluorescein from Eastman-Kodak Co. (Rochester, N.Y.). Phosphatidylcholine is isolated from egg yolk according to Pangborn (1951) and phosphatidylserine from brain according to Sanders (1967). Phospholipids are isolated from pig pancreas according to the method of Folch et al. (1957).

3.2.2. Preparation of liposomes

The procedure for isolation of acinar cells and the incubation medium have been described in Chapter V.

Liposomes are prepared in the following way: A solution of the desired phospholipids in organic solvent is transferred to a round-bottom flask, and the solvent is removed in a rotating evaporator. This results in a thin phospholipid film on the wall of the flask. An aqueous solution, containing the compound to be enclosed in the liposomes, is added (ca 3 ml/20 mg phospholipid). The flask is then shaken for 30 min on a Griffin Shaker. The resulting suspension is sonicated intermittently in a Branson B12 sonifier with microtip at half-maximal output under cooling in ice, until a bluish translucent solution is obtained. The whole procedure is carried out under nitro-

gen to prevent oxidation of the phospholipids. The liposome suspension is centrifuged for 15 min in a minicentrifuge to remove large aggregates and titanium fragments. To check whether there are large multilamellar vesicles present, the liposome suspension is passed over a Sepharose CL-4B column. Large multilamellar vesicles traverse the column rapidly in the void volume, and are thus removed from the small unilamellar vesicles, the liposomes. The fraction of large multilamellar vesicles appears to be negligible. The liposome suspension is passed over a Sephadex G100 column, and is equilibrated and eluted with Krebs-Ringer bicarbonate medium without glucose and albumin (see section 2.2.4). In this medium the liposomes are incubated with the isolated acinar cells.

3.2.3. Incubation of acinar cells with liposomes

To a preparation of isolated acinar cells (10^6 cells/ml) a liposome suspension is added to a final concentration of 1 mM phospholipid. The mixture is incubated under continuous shaking in an O_2/CO_2 (95%/5%) atmosphere. Two procedures are used to check whether the liposome content is transferred to the cytoplasm of the acinar cells. 1) Liposomes, prepared in a KCl buffer containing 5 mM Ca^{2+} are incubated with the cells and amylase release is measured. If the liposome content is transferred to the cytoplasm, this should result in an increase in the cytoplasmic calcium concentration and hence in a stimulated enzyme release. 2) A more direct method is incubating acinar cells with liposomes containing 100 mM 6-carboxyfluorescein, which in this high concentration does not fluoresce. Release of the liposome content into the acinar cell would result in dilution of the 6-carboxyfluorescein and thus lead to fluorescence (Weinstein et al., 1977). After removal of the liposome containing medium by sedimentation and washing, the fluorescence in the cells is measured. Excitation and emission maxima are at 460 and 530 nm, respectively.

3.3. Results

3.3.1. Incubation of acinar cells with liposomes

Table 3.1. INCUBATION OF ACINAR CELLS WITH PC: CHOLESTEROL: PS (5:4:1) LIPOSOMES CONTAINING Ca^{2+}

	Amylase release	
Incubation time (min)	Control (%)	+ liposomes (%)
15	3.0	1.9
30	4.1	4.1
45	4.6	4.5
60	7.3	5.1

Acinar cells are incubated in the absence and presence of PC: cholesterol: PS (5:4:1) liposomes containing 5 mM Ca^{2+}. Amylase activity is measured in samples of the supernatant and expressed as percent of the total amount of amylase present in the cells.

Acinar cells are incubated in the presence of liposomes of various compositions: neutral liposomes consisting only of PC, negatively charged liposomes consisting of PC and PS (9:1) or of PC, cholesterol and PS (9:1:1 and 5:4:1) and positively charged liposomes consisting of PC and stearylamine (9:1) or of PC, cholesterol and octadecylamine (5:4:1). Table 3.1 shows the result of an incubation of acinar cells with PC:cholesterol:PS (5:4:1) liposomes containing 5 mM Ca^{2+}. It is clear that the amylase release is not stimulated by the liposomes, which indicates that there is no transfer of the liposome content into the cell. In a parallel experiment acinar cells are incubated with PC:cholesterol:PS (5:4:1) liposomes containing 6-carboxyfluorescein. Table 3.2 shows that after 60 min incubation no fluorescence can be detected in the cells, even after addition of Triton X-100. This shows that the liposome content is not incorporated by the cells. As expected, the fluorescence of the medium containing the liposomes is greatly enhanced by the addition of Triton X-100, due to the approximate 10^4-fold dilution of the fluorescent

Table 3.2. INCUBATION OF ACINAR CELLS WITH PC:CHOLESTEROL:PS (5:4:1) LIPOSOMES CONTAINING 6-CARBOXYFLUORESCEIN

	Fluorescence (arbitrary units)	
	- Triton X-100	+ Triton X-100
Incubation medium	9.5	170
Cells	not detectable	not detectable

Acinar cells are incubated for 60 min in the presence of PC:cholesterol:PS (5:4:1) liposomes containing 6-carboxyfluorescein. The incubation medium and the cells are then separated by centrifugation and the fluorescence is measured in both fractions, with and without addition of 1% Triton X-100 (excitation maximum 460 nm, emission maximum 530 nm).

compound. Incubation of acinar cells with liposomes of other compositions gives similar results. The liposome concentration in the incubation medium has been varied between 0.3 and 2.2 mM vesicle lipid. This has no effect on the results. A higher liposome concentration does not seem to be useful, for at a concentration of 1-2 mM vesicle lipid saturation of the interaction of liposomes with cells occurs (Van Renswoude, A.J.B.M., Hoekstra, D. and Scherphof, G.L., personal communication).

Because there are indications that serum inhibits the uptake of lipid vesicles by cells (Hoekstra et al., 1979), we have carried out the incubations of cells and liposomes in the presence and absence of bovine serum albumin. In either case the results are negative with both test methods.

The above incubations have all been carried out at 37 ^{o}C. However, Weinstein et al. (1979) reported that at 42 ^{o}C the transfer of liposome content to tumor cells is stimulated 4-fold. Thus we have raised the temperature of incubation to see whether this has a pos-

itive effect on the incorporation of PC:cholesterol:PS (5:4:1) liposomes by the cells. It appears that the temperature can be raised to 46 ^{o}C without harmful effects on the acinar cells (as judged by light microscopy and amylase release), but at this high temperature there is again no transfer of liposome content to the cells.

In vivo experiments have shown that liposomes composed of saturated phospholipids are more effective than those consisting of highly unsaturated phospholipids (Alvin and Steck, 1979). However, when we replace the highly unsaturated PC in PC:cholesterol:PS (5:4:1) liposomes by the saturated dimyristoyl-PC the results are still negative.

Finally, we have tried whether liposomes consisting of phospholipids isolated from pig pancreas are more suited to be taken up by the acinar cells. However, these experiments also gave negative results.

3.4. Discussion

There are several reports showing that it is possible to introduce substances into cells, both in vivo and in vitro, by means of liposomes (Poste et al., 1976; Pagano and Weinstein, 1978). Theoharides and Douglas (1978) and Gutman et al. (1979) have used this technique to obtain more information about the substances involved as second messengers in stimulus-secretion coupling in mast cells and adrenal medulla, respectively. We have tried to apply this technique on pancreatic acinar cells. However, although we have varied both the composition of the liposomes and the incubation conditions, we have not been able to find evidence for transfer of the liposome content (Ca^{2+} or 6-carboxyfluorescein) into the acinar cells. It is possible that we have not found the right conditions for the incorporation of liposomes by the acinar cells. Another possibility is that the pancreatic acinar cells have some properties which make them less suitable for interaction with liposomes. So, although the technique has been used with success on other cells, the results with pancreatic acinar cells have so far been negative.

CHAPTER IV

EFFECT OF EXTRACELLULAR CALCIUM ON PANCREOZYMIN-INDUCED CYCLIC AMP FORMATION

4.1. Introduction

Pancreozymin stimulates the secretion of digestive enzymes by the acinar cells of the pancreas. The mechanism of this stimulation process is not yet clear. On the one hand, there is abundant evidence that pancreozymin causes an increase in the cytoplasmic calcium level of the acinar cell (Case and Clausen, 1973; Gardner et al., 1975; Schreurs et al., 1976a; Kondo and Schulz, 1976a). On the other hand, there is clear evidence for the existence of a pancreozymin-stimulated adenylate cyclase in the acinar cell (Svoboda et al., 1976, 1978; Kempen et al., 1977a; De Pont et al., 1979). However, there is conflicting evidence for a pancreozymin-induced increase in the cyclic AMP level. While Case et al. (1972) measure a 10-fold, transient increase in cyclic AMP content, other investigators find no increase (Benz et al., 1972; Robberecht et al., 1974; Albano et al., 1976) or only a small increase (Deschodt-Lanckman et al., 1975; Kempen et al., 1977a).

It occurred to us that the increase in the cytoplasmic calcium level might antagonize the formation of cyclic AMP. Such an effect would not show up in experimental measurements of the adenylate cyclase activity, since these are carried out with broken cell preparations or crude membrane fractions. It could, however, affect experimental measurements of the cyclic AMP level, where incubation of intact cells is used.

This has led us to determine the effect of lowering the external calcium concentration on the cyclic AMP level of rat pancreas fragments, incubated in the presence of pancreozymin-C-octapeptide.

4.2. Materials and methods

4.2.1. Materials

The cyclic AMP binding protein is isolated from bovine adrenal cortex according to the method described by Brown et al. (1971). Pancreozymin-C-octapeptide was synthesized by Dr. H.M.Rajh (Departments of Organic Chemistry and Biochemistry, University of Nijmegen, Nijmegen, The Netherlands). Cyclic [^{3}H]AMP (38.5 Ci/mmol) is purchased from the Radiochemical Centre (Amersham, U.K.). 1-Methyl-3-isobutylxanthine is obtained from Aldrich-Europe (Beerse, Belgium), bovine serum albumin from Sigma Chemical Co. (St.Louis, Mo., U.S.A.), cyclic AMP from Boehringer (Mannheim, G.F.R.), Dowex 50W-X4 (200-400 mesh) from Fluka (Buchs, Switzerland), activated charcoal (Norit SX-1) from Norit (Amersfoort, The Netherlands) and Pico-fluor TM from Packard (Brussels, Belgium). All other chemicals are of reagent grade.

4.2.2. Tissue preparation and incubation

Male and female Wistar rats, weighing 150-200 g and fed ad libitum, are killed by a blow on the neck, followed by carotic exsanguination. The pancreas is immediately removed, freed of fat and mesentery and cut in fragments of 10-20 mg wet weight. The (pre-)-incubation medium consists of Krebs-Ringer bicarbonate buffer (see section 2.2.4), containing 10 mM 1-methyl-3-isobutylxanthine. The calcium concentration of the medium is varied between 0 and 2.5 mM.

Fragments are pre-incubated for 20 min (37 ^{o}C) in 500 µl medium, followed by incubation in the presence of pancreozymin-C-octapeptide. During each incubation the medium is gassed with a mixture of 95% O_2 and 5% CO_2. The last incubation is terminated by addition of 100 µl ice-cold 50% trichloroacetic acid, immediately followed by homogenization in a Potter-Elvehjem tube. This homogenate is used for the cyclic AMP determination.

4.2.3. Determination of cyclic AMP

Cyclic AMP is determined by the method used by Kempen et al.

(1977a): 0.2 µCi cyclic [^{3}H]AMP is added to the homogenate, which is then centrifuged to remove the precipitated material. The supernatant is freed of trichloroacetic acid by repeated extraction with water-saturated diethylether. The extract is further purified by means of a Dowex-50W column according to Krishna et al. (1968). The cyclic AMP content of the extract is determined by saturation assay with cyclic AMP binding protein as described by Brown et al. (1971). In each experiment a standard curve is made by adding 0.2 µCi cyclic [^{3}H]AMP to known amounts of cyclic AMP and treating these samples in the same way as the tissue samples. The values for the cyclic AMP content are expressed in pmol/mg protein.

4.2.4. Assay methods

Protein concentration is measured according to Lowry et al. (1951) using bovine serum albumin as a standard. Radioactivity is counted in a Philips liquid scintillation analyzer, using Pico-fluor TM as scintillation fluid.

4.3. Results

4.3.1. Effect of 1-methyl-3-isobutylxanthine concentration on cyclic AMP level of rat pancreatic fragments

In all experiments 1-methyl-3-isobutylxanthine has been used in order to inhibit the phosphodiesterase activity. In view of the limited solubility of this compound, we have determined the minimal level needed for maximal retention of cyclic AMP. Fig.4.1 gives the cyclic AMP contents of fragments, incubated during 20 min in Ca^{2+}-free medium in the presence and absence of 10^{-6}M pancreozymin-C-octapeptide and varying concentrations of 1-methyl-3-isobutylxanthine. The stimulatory effect of the peptide is maximal at 10 mM 1-methyl-3-isobutylxanthine, while without pancreozymin-C-octapeptide there is no significant increase in cyclic AMP level at this 1-methyl-3-isobutylxanthine concentration. For this reason we have used a 10 mM concentration in all further experiments.

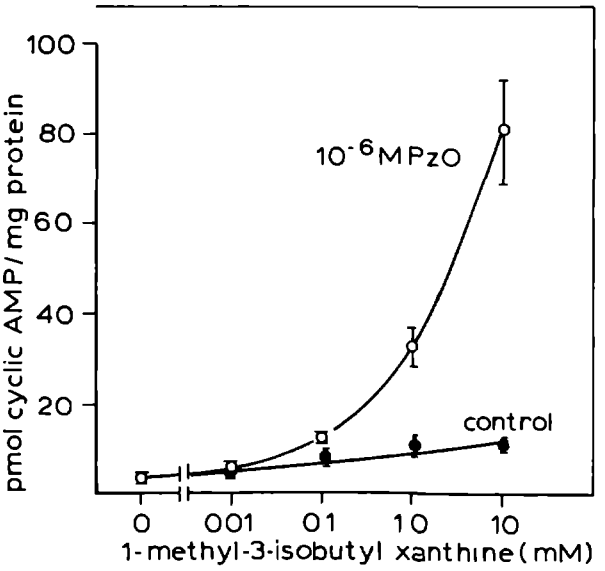


Figure 4.1

Effect of 1-methyl-3-isobutylxanthine concentration on cyclic AMP levels in rat pancreatic fragments. The fragments are incubated for 20 min in the presence (-o-) or absence (-●-) of 10^{-6} pancreozymin-C-octapeptide and varying 1-methyl-3-isobutylxanthine concentration. Means with S.E. of three separate experiments.

4.3.2. Effect of pancreozymin-C-octapeptide on the cyclic AMP level in the presence or absence of calcium

Earlier results from our laboratory show that a 10-min incubation with 3.10^{-7}M pancreozymin-C-octapeptide, in the presence of 2.5 mM Ca^{2+} and 10 mM 1-methyl-3-isobutylxanthine, causes a 1.3-fold increase of the cyclic AMP level of rat pancreatic fragments (Kempen et al., 1977a). We now find that under these conditions after 20 min incubation the cyclic AMP level increases 2.6-fold (Table 4.1).

Table 4.1. EFFECT OF PANCREOZYMIN-C-OCTAPEPTIDE ON CYCLIC AMP LEVEL IN RAT PANCREATIC FRAGMENTS

	Krebs-Ringer bicarbonate 2.5 mM Ca^{2+}	Krebs-Ringer bicarbonate 0 mM Ca^{2+}	
Control	10±1.3 (9)	8.7±0.9 (16)	n.s.
3.10^{-7}M PzO	26±3.2 (4)	43±2.4 (4)	$p < 0.01$
10^{-6}M PzO	37±4.6 (7)	66±5.2 (12)	$p < 0.001$

Rat pancreatic fragments are incubated for 20 min in the presence of 10 mM 1-methyl-3-isobutylxanthine alone (control) or together with pancreozymin-C-octapeptide (PzO) at the indicated concentration, either in the presence or absence of calcium.
Values represent means with S.E. in pmol cAMP/mg protein and in parentheses the number of experiments is given.
P-values are determined by means of the Student t-test (n.s.: not significant).

By omitting the calcium ions from the incubation medium, the stimulating effect of pancreozymin-C-octapeptide on the cyclic AMP content is nearly twice as high. Reduction in the pre-incubation time from 20 to 0 min appears to have little effect on the results, but in all experiments a pre-incubation time of 20 min has been used.

Fig. 4.2 shows dose-response curves for the effect of pancreozymin-C-octapeptide on the cyclic AMP level in rat pancreatic fragments in the presence and absence of calcium. In normal Krebs-Ringer bicarbonate medium, containing 2.5 mM Ca^{2+} and 10 mM 1-methyl-3-isobutylxanthine, the effect of pancreozymin-C-octapeptide is half-maximal at 10^{-6}M and nears a maximal value of 92±1.6 pmol cAMP/mg protein at 10^{-5}M. In calcium-free Krebs-Ringer bicarbonate medium (containing 0.1 mM EGTA and 10 mM 1-methyl-3-isobutylxanthine) the

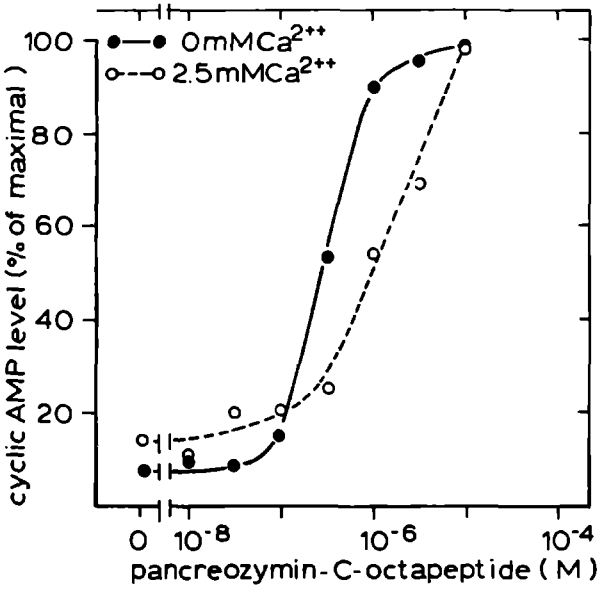


Figure 4.2.

Dose-response curves for pancreozymin stimulation of cyclic AMP formation. Rat pancreatic fragments are incubated for 20 min in the presence of pancreozymin-C-octapeptide and 10 mM 1-methyl-3-isobutylxanthine with or without Ca^{2+}. The cyclic AMP level after incubation with 10^{-5}M pancreozymin-C-octapeptide is taken as 100. Values are means of three (0 mM Ca^{2+}) and two (2.5 mM Ca^{2+}) separate experiments.

dose-response curve is shifted to lower concentrations of pancreozymin-C-octapaptide with half-maximal stimulation at 3.10^{-7}M and reaching a maximal value of 83 $\pm$ 8.9 pmol cAMP/mg protein at 10^{-6}M, which is not exceeded at 10^{-5}M pancreozymin-C-octapeptide. The maximal cyclic AMP level reached in calcium-free medium is the same as that in the normal medium with 2.5 mM Ca^{2+}. Table 4.1 shows that the increase in cyclic AMP level in the presence of 3.10^{-7}M and 10^{-6}M

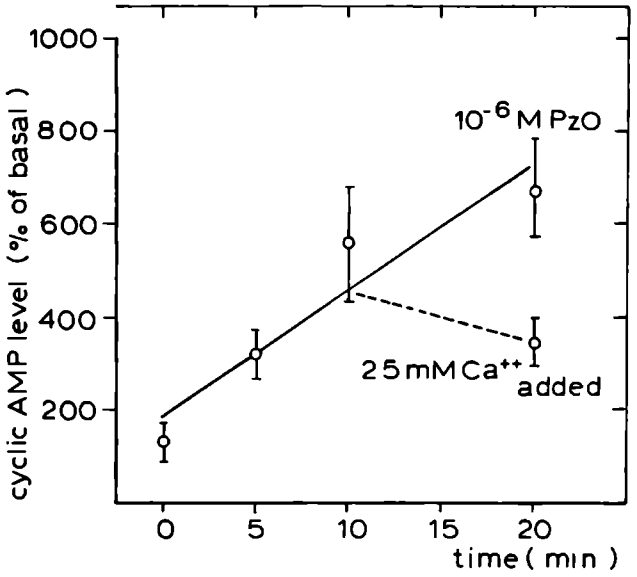


Figure 4.3.

Effect of later addition of calcium on the cyclic AMP level. Rat pancreatic fragments are incubated in the presence of 10^{-6}M pancreozymin-C-octapeptide and 10 mM 1-methyl-3-isobutylxanthine in calcium-free medium. To one fragment 2.5 mM Ca^{2+} is added after 10 min incubation in calcium-free medium. Means with S.E. of four separate experiments.

pancreozymin-C-octapeptide is about twice as high in calcium-free medium than in normal Krebs-Ringer bicarbonate medium.

4.3.3. Effect of later addition of calcium on pancreozymin-stimulated cyclic AMP formation

Incubation of rat pancreatic fragments in calcium-free Krebs-Ringer bicarbonate medium containing 10 mM 1-methyl-3-isobutyl-

xanthine and 10^{-6}M pancreozymin-C-octapeptide produces a nearly linear increase of the cyclic AMP content with time during the first 20 min (Fig.4.3). If after 10 min incubation of a fragment in a calcium-free medium 2.5 mM Ca^{2+} is added, the cyclic AMP level of this fragment does not rise any further during an additional 10-min incubation. The final level of the cyclic AMP content after such a combined incubation (48 $\pm$ 2.6 pmol/mg protein) is significantly lower than after a 20-min incubation in a calcium-free medium (65 $\pm$ 6.6 pmol/mg protein). This means that addition of calcium blocks the formation of cyclic AMP.

4.3.4. Effect of varying the calcium concentration on pancreozymin-stimulated cyclic AMP formation

We have checked whether complete removal of calcium from the medium is required for full enhancement of the pancreozymin-stimulated formation of cyclic AMP. Fig.4.4 shows the cyclic AMP content of fragments after 20 min incubation in media containing 10^{-6}M pancreozymin-C-octapeptide and 10 mM 1-methyl-3-isobutylxanthine with calcium concentrations varied from 0 to 4.5 mM. It is clear that it suffices to lower the calcium concentration to 1.5 mM in order to obtain maximal stimulation. In media with 3.5 or 4.5 mM Ca^{2+} the effect of the peptide on the cyclic AMP content is the same as in normal Krebs-Ringer bicarbonate medium (2.5 mM Ca^{2+}). So there seems to be a critical calcium concentration at about 1.5 mM. If the calcium concentration of the medium is below this value, the stimulation of the adenylate cyclase by pancreozymin-C-octapeptide results in an appreciable increase of the cyclic AMP level; above this concentration the stimulating effect of the peptide is considerably decreased.

4.4. Discussion

It is generally agreed that cyclic AMP is involved in the secretin-induced secretion of fluid and electrolytes (Case, 1973;

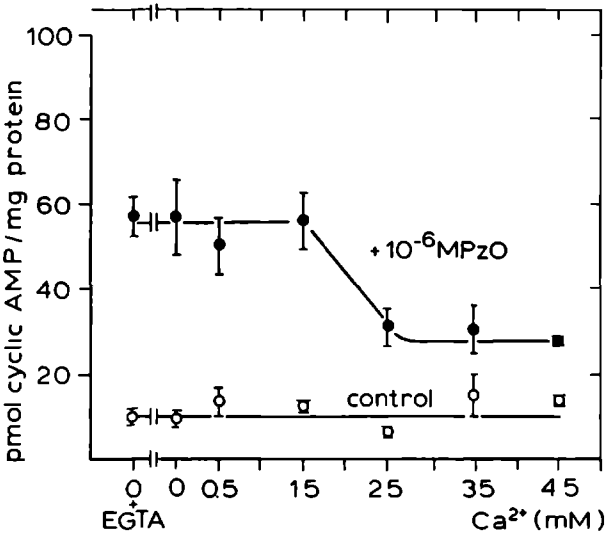


Figure 4.4.

Effect of varying the Ca^{2+} concentration on pancreozymin stimulation of cyclic AMP formation. Rat pancreatic fragments are incubated for 20 min in the presence of 10^{-6}M pancreozymin-C-octapeptide and 10 mM 1-methyl-3-isobutylxanthine. Means with S.E. of five separate experiments.

Robberecht et al., 1974, 1976), but a role of cyclic AMP in pancreozymin-induced enzyme secretion is still questionable. Incubation with cyclic AMP or its analogues causes a slight increase in the enzyme release from several pancreatic preparations, but this increase is very small compared to the stimulation of the enzyme secretion by pancreozymin or acetylcholine (Benz et al., 1972; Heisler et al., 1972; Kempen et al., 1977a).

The fact that pancreozymin-C-octapeptide stimulates the adenylate cyclase activity (Svoboda et al., 1976, 1978; Kempen et al., 1977a; De Pont et al., 1979) suggests a role of cyclic AMP in the

enzyme secretion process. However, a rise in cyclic AMP level under the influence of this hormone derivative has only been measured in a few cases (Case et al., 1972; Deschodt-Lanckman et al., 1975; Kempen et al., 1977a), while other investigators do not find this effect (Benz et al., 1972; Robberecht et al., 1974; Albano et al., 1976). It occurred to us that this discrepancy may be due to the fact that pancreozymin-C-octapeptide not only stimulates the adenylate cyclase of the acinar cell, but also causes an increase in its cytoplasmic calcium concentration (Case and Clausen, 1973; Gardner et al., 1975; Schreurs et al., 1976a; Kondo and Schulz, 1976a). The latter effect might antagonize the stimulating effect on the adenylate cyclase.

In order to test this hypothesis we have tried to influence the cytoplasmic calcium level of the acinar cell by varying the extracellular calcium concentration, and then measuring the effect of pancreozymin-C-octapeptide on the cellular cyclic AMP level. These experiments have been carried out with rat pancreatic fragments, which consist for 78% of acinar cells on a volume basis (Hegre et al., 1972). It seems likely that pancreozymin-C-octapeptide acts on the acinar cells, since ductular cells have no receptor for pancreozymin (Milutinovic et al., 1977) and pancreozymin does not increase the cyclic AMP content in rat pancreatic duct fragments, obtained after feeding rats with a copper-deficient diet (Fölsch et al., 1977).

In measuring cyclic AMP and its formation in the cell, it is necessary to inhibit phosphodiesterases which convert it to 5'AMP. For this purpose some investigators use theophylline. Kempen et al. (1977a) have shown, however, that 1-methyl-3-isobutylxanthine is a more potent inhibitor of phosphodiesterase than theophylline. This may explain, why in all cases where 5 mM or less theophylline has been used, no pancreozymin-induced increase of the cyclic AMP level is found (Benz et al., 1972; Robberecht et al., 1974; Albano et al., 1976) and with 10 mM theophylline only a small increase is observed (Deschodt-Lanckman et al., 1975), smaller than we have previously found with 10 mM 1-methyl-3-isobutylxanthine (Kempen et al., 1977a). It must be admitted that the 10 mM 1-methyl-3-isobutylxanthine con-

centration is rather high and might cause some other effects in addition to the inhibition of cyclic AMP phosphodiesterase, but, as shown in Fig.4.1, this concentration was necessary to obtain maximal stimulation of the cyclic AMP level by pancreozymin.

In confirmation of our hypothesis for the effect of calcium on the pancreozymin-induced increase in cyclic AMP level, we observe a considerably larger effect (7.6-fold) in the absence of calcium than with 2.5 mM calcium (3.7-fold), both in the presence of 10^{-6}M pancreozymin-C-octapeptide and 10 mM 1-methyl-3-isobutylxanthine. This observation suggests that the stimulatory effect of pancreozymin-C-octapeptide on the adenylate cyclase activity may be antagonized by the effect of the hormone on the cytoplasmic calcium concentration. The same phenomenon has been observed in fly salivary gland by Prince et al. (1972), who find that the stimulating effect of 5-hydroxytryptamine on the cyclic AMP content is enhanced in calcium-free medium.

In our preparation it actually suffices to lower the calcium concentration from 2.5 mM, which is the concentration, used by the investigators who measure no or a small pancreozymin-induced rise in cyclic AMP level, to 1.5 mM to achieve maximal stimulation by the hormone. It is important to note that the free calcium concentration in rat plasma is about 1.5 mM, since the total plasma calcium concentration in this species is 3 mM (Cole et al., 1944), 50% of which is protein-bound (Blaustein, 1974). It is unlikely that the extracellular calcium concentration influences the adenylate cyclase activity directly, but probably it affects the cytoplasmic calcium concentration, which then influences the stimulation of adenylate cyclase. The assumption that the cytoplasmic calcium concentration is influenced by the extracellular calcium concentration is supported by the observation that in acinar cells of the rabbit pancreas the amount of exchangeable calcium decreases when the calcium concentration of the incubation medium is lowered (Renckens et al., 1978).

It is possible that the adenylate cyclase activity is directly inhibited by a rise in cytoplasmic calcium concentration. Rutten et al. (1972) have indeed reported inhibition of the activity of a

pancreatic adenylate cyclase preparation by calcium ($\geq$ 1 mM) in vitro, while Stolc (1979) found that cyclic AMP formation in intact granulocytes is inhibited when the intracellular calcium concentration is raised by adding ionophore A23187 and 5 mM Ca^{2+} to the medium. However, these inhibitory effects occur at calcium concentrations, which are far higher than the expected cytoplasmic calcium concentration, even after stimulation by pancreozymin. So a direct inhibition of the adenylate cyclase activity by the rise in cytoplasmic calcium concentration is not very likely. Another possibility is that the rise in cyclic GMP level, which follows the increase in cytoplasmic calcium concentration upon stimulation (Robberecht et al., 1974; Albano et al., 1976; Kapoor and Krishna, 1977; Lopatin and Gardner, 1978) would lead to the inhibition of adenylate cyclase activity. However, no direct inhibitory effect of cyclic GMP on adenylate cyclase activity in a rat pancreatic particulate fraction can be observed (Renckens et al., unpublished observation). This means that the regulation of the adenylate cyclase activity by calcium takes place via another, as yet unknown pathway.

These findings still leave unanswered the question whether cyclic AMP plays a second messenger role in the stimulation of enzyme secretion or whether its formation is a secondary effect of pancreozymin on the acinar cell. However, they do seem to have resolved the controversy over whether or not pancreozymin is able to raise the cyclic AMP level in the pancreatic acinar cell.

CHAPTER V.

CALCIUM MOVEMENTS IN ISOLATED ACINAR CELLS OF RABBIT PANCREAS

5.1. Introduction

There are strong indications that Ca^{2+} ions play a role as second messenger in the pancreatic enzyme secretion. Stimulation of the enzyme secretion causes a rise in the cytoplasmic calcium concentration (Case and Clausen, 1973; Williams and Lee, 1974; Schreurs et al., 1976a). This increase may either be caused by a release of calcium from an intracellular pool or by an increased calcium influx under influence of the stimulant. The first possibility is supported by the observation that addition of a secretagogue to various pancreatic preparations, which are preloaded with $^{45}Ca^{2+}$, leads to an increased $^{45}Ca^{2+}$-efflux (Case and Clausen, 1973; Schreurs et al., 1976a; Gardner et al., 1975). On the other hand, Heisler and Grondin (1973) and Kondo and Schulz (1976a, 1976b) measure an increased influx of $^{45}Ca^{2+}$ under the influence of a stimulant.

Isolated acinar cells form the most suitable preparation for measuring $^{45}Ca^{2+}$-fluxes, for such a preparation contains only a minor (5%) fraction of non-acinar cells and the poorly accessible extracellular spaces around the cells are negligible. However, even experiments with isolated acinar cells give conflicting results: Gardner et al. (1975) find a $^{45}Ca^{2+}$-efflux, while under different conditions Kondo and Schulz (1976b) observe an increased influx of $^{45}Ca^{2+}$ upon addition of a stimulant to isolated acinar cells. We have measured $^{45}Ca^{2+}$-fluxes and the calcium distribution in isolated acinar cells of the rabbit pancreas under various conditions to obtain more information about the role of calcium in stimulus-secretion coupling.

5.2. Materials and methods

5.2.1. Materials

$^{45}CaCl_2$ (800 mCi/mmol), ^{3}H-leucine (1Ci/mmol) and ^{3}H-inulin (900 mCi/mmol) are purchased from the Radiochemical Centre (Amersham, England). Aquasol is supplied by New England Nuclear (Boston, Mass., U.S.A.), carbachol is obtained from Brocades-AFC (Holland), collagenase (153 U/mg) from Worthington Biochemical Corp.(Freehold, N.Y., U.S.A.). Hyaluronidase, trypsin inhibitor and bovine serum albumin are purchased from Sigma Chemical Co. (St.Louis, Mo., U.S.A.). All other chemicals are of reagent grade.

5.2.2. Tissue preparation

Male and female New Zealand white rabbits, weighing 2-3 kg, are fasted overnight and are killed by a blow on the neck followed by carotic exsanguination.The pancreas is removed and is freed of fat and mesentery. Isolated acinar cells are prepared according to the method described by Amsterdam and Jamieson (1974). This method is based on consecutive incubations with collagenase and hyaluronidase, EDTA and again with collagenase and hyaluronidase. After this digestion mild shearing is used to disperse the tissue, the suspension is filtered through nylon gauze and purified by centrifugation through a 4% (w/v) albumin layer. Comparison of the number of cells in a cell suspension, determined in a counting chamber, and the protein content of the suspension shows that one mg protein corresponds to 2.1 x 10^6 cells (S.E. 0.23, n=5). When in an experiment the protein secretion must be measured, the cell proteins are labeled with ^{3}H-leucine by incubation (15 min, 37 ^{o}C) of the pancreas in a medium containing carrier-free ^{3}H-leucine (50 µCi) and an amino acid mixture without leucine (Eagle, 1959) before the isolation of the cells.

5.2.3. Incubation medium

The cells (10^7 cells/ml) are preincubated and incubated in a Krebs-Ringer bicarbonate medium (see section 2.2.4) to which is added trypsin inhibitor (0.2 mg/ml) and bovine serum albumin (1% w/v). The pH of the medium is adjusted to 7.4 with HCl. During the incubation

it is kept at 37 ^{o}C and is constantly gassed with an O_2/CO_2 mixture (95%, 5%). Variations in the composition of the incubation medium are indicated in the text.

5.2.4. ^{3}H-labeled protein secretion

The secretion of digestive enzymes is determined by measuring the release of TCA-insoluble ^{3}H-protein. The results obtained in this way agree very well with those obtained by measuring the release of α-amylase (not shown). A 250-µl sample of suspension is centrifuged for 5 sec in a microcentrifuge. To each of two 100-µl aliquots of the supernatant 5 ml ice-cold 10% TCA is added. After centrifugation the pellets are washed once with 5 ml ice-cold 10% TCA and dissolved in 1 ml 0.2 M NaOH-solution; 500-µl aliquots of these solutions are dispersed in 10 ml Aquasol and are counted for radioactivity. The results are expressed as percent of the total TCA-insoluble ^{3}H-protein content of medium and cells together.

5.2.5. Transport of $^{45}Ca^{2+}$

After 60 min preincubation the cells are centrifuged (150 x g, 5 min) and resuspended in 5 ml medium. At time zero 12.5 µCi $^{45}Ca^{2+}$ is added. When more than one pancreas is used, the quantities of medium and $^{45}Ca^{2+}$ are adjusted. Every 10 minutes a 50-µl aliquot of the cell suspension is taken and washed to remove extracellular $^{45}Ca^{2+}$. The cells are washed by filtration instead of by centrifugation. The advantages of filtration are: 1. sampling is faster, allowing more samples to be processed in a given period of time, and 2. lower basal $^{45}Ca^{2+}$ values are obtained, allowing detection of small differences in $^{45}Ca^{2+}$ content. Several media and filtration procedures for washing the cells have been tried. The following procedure gives the best results: the sample is added to 6 ml ice-cold wash solution, containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4) and 10 mM EDTA. The mixture is immediately poured onto a glassfiber filter (Schleicher and Schüll, nr.6) and filtered by suction. The cells on the filter are washed once with 6 ml cold wash solution and the filter is placed in 10 ml Aquasol. The protein content of the

suspension is measured, so that the $^{45}Ca^{2+}$ content can be expressed as nmol $^{45}Ca^{2+}$/mg protein (we use the notation $^{45}Ca^{2+}$, although this refers, of course, to the sum of $^{40}Ca^{2+}$ and $^{45}Ca^{2+}$ exchanged during the experiment). The values for the $^{45}Ca^{2+}$ content are corrected for calcium in the interstitial space and calcium adhering to the filter by subtracting from them the value for the $^{45}Ca^{2+}$ content measured at time zero. This value varies from 0.4 nmol $^{45}Ca^{2+}$/mg protein in the medium with 0.1 mM Ca^{2+} to 0.9 nmol $^{45}Ca^{2+}$/mg protein in the medium with 2.5 mM Ca^{2+}.

5.2.6. Total cellular calcium content

The total cellular calcium content is determined according to the method described by Hendriks et al. (1974). A 750-µl aliquot of the cell suspension is centrifuged, the pellet is resuspended in 200 µl water, and 100 µl of this suspension is transferred to a quartz tube. After evaporation of water at 90 °C, destruction is achieved by heating for 0.5 hr at 180 °C with 200 µl of a H_2SO_4-$HClO_4$-HNO_3-mixture (1:3:12, by vol.).Then 2 ml 0.5% $LaCl_3$ in 0.1 M HCl is added and the calcium is measured by atomic absorption spectrophotometry.

A correction is made for extracellular calcium by measuring the inulin space of the pellet. For this purpose 2 µCi ^{3}H-inulin is added to the cell suspension 5 min before sampling, and a 750-µl aliquot of the suspension is centrifuged. The pellet is resuspended in 200 µl water, and the radioactivity of 100 µl suspension and 100 µl supernatant are measured, so that the inulin space in the pellet can be calculated. By measuring the calcium concentration in the supernatant, the amount of extracellular calcium in the pellet can be calculated. By subtracting this value from the total calcium content in the pellet, the amount of intracellular calcium is known (including any calcium tightly adhering to the outside of the cell membrane). The amount of extracellular calcium in the pellet is 7% in the medium with 0.1 mM Ca^{2+}, 12% in the medium with 0.5 mM Ca^{2+} and 35% in the medium with 2.5 mM Ca^{2+}.

5.2.7. Assay methods

Protein is determined according to Lowry et al. (1951), using bovine serum albumin as a standard.

Lactic dehydrogenase is assayed with the technique of Kubowitz et al. (1943) and amylase with the technique of Bernfeld (1955).

Radioactivity is counted in a Philips liquid scintillation analyzer. In double-labeling experiments the radioactivity of each isotope is calculated by means of the external standard ratio method.

5.3. Results

5.3.1. Viability of the cells

Light microscopy of the cell suspension shows that less than 5% of the cells are non-acinar cells and that the large majority of the cells is capable of excluding trypan blue. If more than 10% of the cells in a preparation take up trypan blue, the preparation is discarded.

The protein secretion of the cells can be stimulated by carbachol. Immediately after addition of 10^{-5} M carbachol to the incubation medium, there is an increase in protein secretion. The amount of enzyme secreted during the first 10 min after addition of carbachol is 3.9 (SE 0.22, n=5) times the amount of enzyme secreted during the last 10 min before addition of carbachol. The enzyme secretion gradually decreases with time, but remains above the control level during the entire experiment of up to 3 hrs (Fig. 5.1). Hence, isolated acinar cells appear to be a suitable preparation for studying the stimulus-secretion coupling in the pancreas.

5.3.2. Choice of sampling technique

Filtration rather than centrifugation is used to measure the $^{45}Ca^{2+}$ uptake. Several media and procedures have been tested for washing the cells. The following washing media have been used: Krebs-Ringer bicarbonate with and without 10 mM EDTA, and the medium used by Shelby et al. (1976) containing 150 mM NaCl, 10 mM Tris-HCl (pH

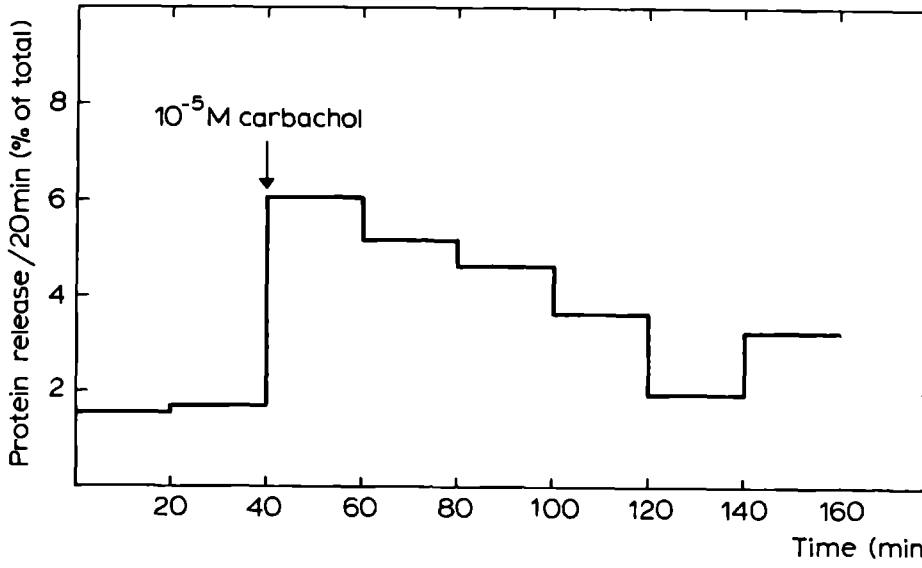


Figure 5.1.

Effect of 10^{-5} M carbachol on the secretion of ^{3}H-protein from isolated acinar cells. Representative for four experiments.

7.4) and 10 mM EDTA. The cells have been washed by three different procedures: a) the sample is added to 6 ml washing medium, another 6 ml washing medium is added and the mixture is immediately poured over a filter; b) the sample is added to 6 ml washing medium, this mixture is filtered and the cells on the filter are washed once with 6 ml washing medium; c) the sample is treated as in procedure b, but the cells on the filter are washed twice with 6 ml washing medium. The results are presented in Table 5.1.

With normal Krebs-Ringer bicarbonate the radioactivity on the filter is much higher than after washing with the other two media, suggesting that Krebs-Ringer bicarbonate without EDTA does not completely remove extracellular calcium even after washing the cells twice. With the other media the radioactivity on the filter remains

Table 5.1. *$^{45}Ca^{2+}$ AND ^{3}H-PROTEIN LEFT ON THE FILTER*

Washing medium	Krebs-Ringer medium		Krebs-Ringer medium + 10 mM EDTA		150 mM NaCl, 10 mM EDTA 10 mM Tris-HCl (pH 7.4)	
Washing procedure	$^{45}Ca^{2+}$ x10^3 cpm	^{3}H-protein x10^3 cpm	$^{45}Ca^{2+}$ x10^3 cpm	^{3}H-protein x10^3 cpm	$^{45}Ca^{2+}$ x10^3 cpm	^{3}H-protein x10^3 cpm
a	5.1±0.72	30.6±0.51	2.6±0.21	29 ±1.4	2.7±0.13	26±1.6
b	5.3±0.69	26.7±0.93	1.7±0.20	25 ±2.6	1.4±0.12	23±1.7
c	5 ±1.1	23 ±2.5	1.0±0.09	15.9±0.92	1.1±0.17	21±1.1

$^{45}Ca^{2+}$ uptake and ^{3}H-protein content of isolated acinar cells are measured after washing in different media and by different procedures. Washing procedures a, b, and c are described in the text. Mean values with standard error of the mean of four separate determinations in one experiment are given.

in the same range after the second washing. The advantage of the NaCl, Tris-HCl, EDTA medium is that the level of radioactivity does not change any more during the second washing, so that one washing suffices.

The possibility exists that the cells are damaged during the washing procedure and that this damage would cause the difference in the results obtained with normal Krebs-Ringer bicarbonate and the other washing media. To check this we have washed cells, the proteins of which were labeled with ^{3}H-leucine, with the three washing media following the three different procedures. Table 5.1 shows the results of this experiment. Only with Krebs-Ringer bicarbonate containing 10 mM EDTA does the radioactivity on the filter decrease considerably during the second washing, suggesting that part of the cells is damaged during washing in this medium.

The results shown in Table 5.1 have led us to use the NaCl, Tris-HCl, EDTA medium and to wash the cells according to procedure b.

5.3.3. Effect of albumin concentration

One of the differences between the experimental conditions used by Kondo and Schulz (1976a) and those used by Gardner et al. (1975) is the albumin concentration in the incubation medium. We have, therefore, compared the $^{45}Ca^{2+}$ uptake in an acinar cell preparation in media containing either 1% (w/v) or 0.1% (w/v) albumin. After 90 min incubation in the 1% albumin medium the $^{45}Ca^{2+}$ content of the cells is 2.1 nmol/mg protein (SE: 0.8, n=2), and in the 0.1% albumin medium it is 1.8 nmol/mg protein (SE: 0.1, n=2). So there is no significant difference in the $^{45}Ca^{2+}$ uptake in these media. We have used an albumin concentration of 1% (w/v) in all further experiments, since this concentration has been recommended by Amsterdam and Jamieson (1974) as giving minimal cell lysis.

5.3.4. Effect of Ca^{2+} concentration on the $^{45}Ca^{2+}$ uptake

Another difference between the experimental conditions used by Kondo and Schulz (1976a) and those used by Gardner et al. (1975) is the extracellular calcium concentration during the uptake period.

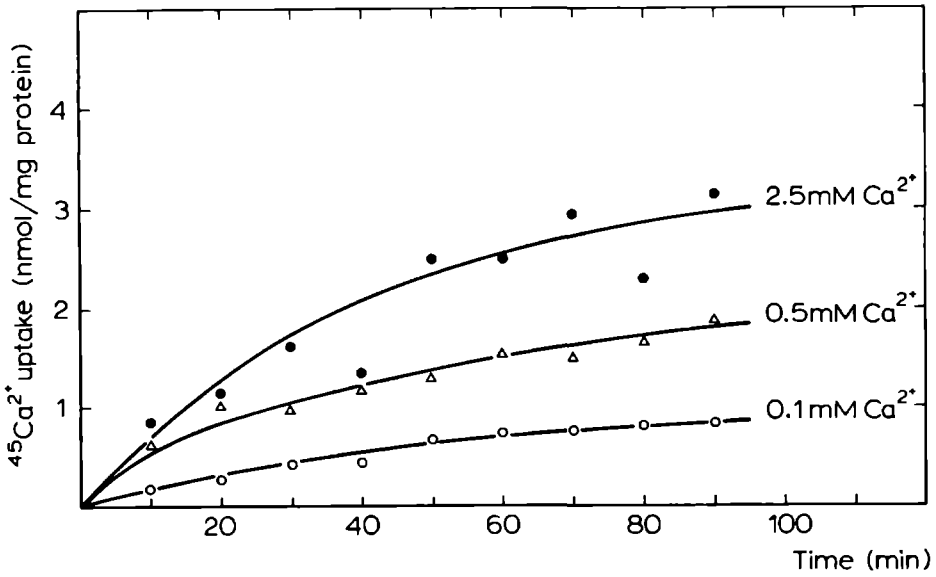


Figure 5.2.

Effect of medium calcium concentration on $^{45}Ca^{2+}$ uptake by isolated acinar cells. Representative for six experiments.

We have, therefore, measured $^{45}Ca^{2+}$ uptake in media with three different Ca^{2+}-concentrations: 0.1 mM, 0.5 mM and 2.5 mM. Fig. 5.2 shows that with increasing Ca^{2+}-concentration in the medium the $^{45}Ca^{2+}$ uptake of the cells increases, which effect has also been observed by Kondo and Schulz (1976b). Table 5.2 (column b) gives the values for the $^{45}Ca^{2+}$ content of the cells after 90 min incubation, when a steady-state has been reached.

5.3.5. Effect of Ca^{2+} concentration on total calcium content

After 90 min incubation in media containing 0.1 mM, 0.5 mM or 2.5 mM Ca^{2+}, the total cellular calcium content has been measured. The results are shown in Table 5.2 (column a). Like the $^{45}Ca^{2+}$

Table 5.2. EFFECT OF MEDIUM Ca^{2+} CONCENTRATION ON THE CELLULAR CONTENTS OF Ca^{2+} AND $^{45}Ca^{2+}$ IN ISOLATED ACINAR CELLS OF RABBIT PANCREAS

Ca^{2+} medium mM	Ca^{2+}-content nmol/mg protein a	$^{45}Ca^{2+}$-content nmol/mg protein b	unexchangeable Ca^{2+} nmol/mg protein a-b
0.1	11.6±1.2 (n=7)	1.0±0.1 (n=5)	10.6 ± 1.2
0.5	13.3±1.8 (n=6)	1.8±0.1 (n=6)	11.5 ± 1.8
2.5	15.3±2.0 (n=5)	3.2±0.3 (n=6)	12.1 ± 2.0

Values, representing means with standard errors of the mean for the indicated numbers of experiments, are measured after 90 min incubation in a medium with the indicated Ca^{2+} concentrations.

content, the total cellular calcium content increases with the calcium concentration of the medium. The results show that only part (9-12%) of the total calcium content has been replaced by $^{45}Ca^{2+}$ after 90 min incubation. By subtracting the values for the $^{45}Ca^{2+}$ content from those for the total calcium content, the amount of non-exchangeable calcium in the cells can be calculated (Table 5.2, column a-b). This amount appears to be independent of the calcium concentration in the medium.

5.3.6. Effect of carbachol on $^{45}Ca^{2+}$ content of the cells

Addition of 10^{-5} M carbachol to cells incubated for 90 min with $^{45}Ca^{2+}$ results in a small decrease of the $^{45}Ca^{2+}$ content of the cells. This suggests that there is a $^{45}Ca^{2+}$ efflux upon stimulation. Fig. 5.3 shows that this effect coincides with the increase in the ^{3}H-protein secretion, when measured in the same experiment.

Fig. 5.4 demonstrates that the reduction of the $^{45}Ca^{2+}$ content

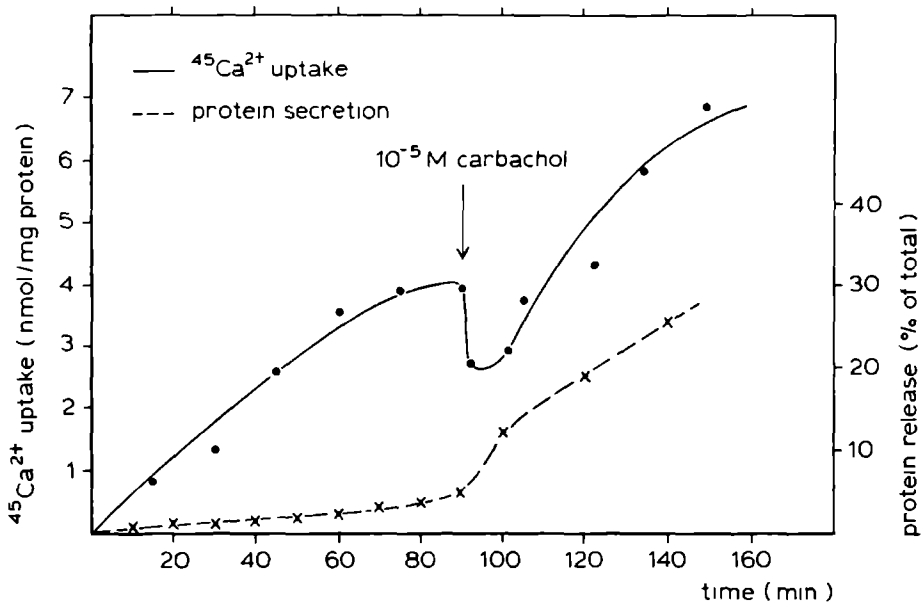


Figure 5.3.

Effect of 10^{-5} M carbachol on ^{3}H-protein secretion and $^{45}Ca^{2+}$ uptake by isolated acinar cells in a medium containing 2.5 mM Ca^{2+}. Representative for two experiments.

occurs at each of the three extracellular calcium concentrations. The magnitude of the reduction is virtually independent of the extracellular calcium concentration, although there is a slight tendency to an increased reduction with increasing calcium concentration. However, the recovery process after stimulation differs considerably with the calcium concentration in the medium. In all three media calcium is taken up again, but only in the medium containing 2.5 mM Ca^{2+} is the $^{45}Ca^{2+}$ content of the cells 60 min after addition of carbachol significantly higher than before stimulation. In Table 5.3 the results of these experiments are summarized.

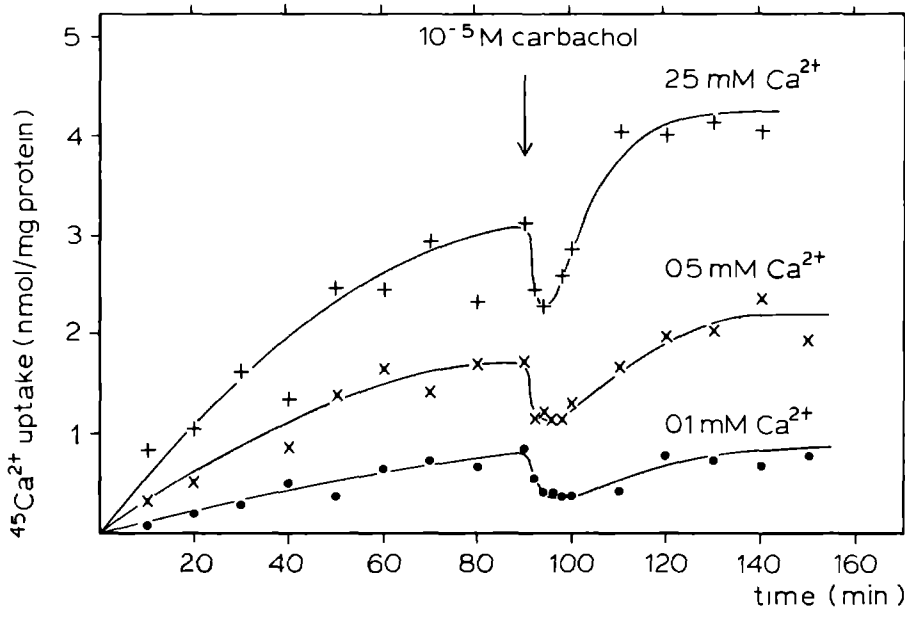


Figure 5.4.

Effect of 10^{-5} M carbachol on $^{45}Ca^{2+}$ uptake by isolated acinar cells in media with different calcium concentrations. Representative for four experiments.

5.3.7. Effect of carbachol on $^{45}Ca^{2+}$ influx

The experiments described in the previous paragraph suggest that at a high extracellular calcium concentration carbachol does not only affect the $^{45}Ca^{2+}$ efflux but also stimulates the influx of $^{45}Ca^{2+}$. These experiments are, however, not very suited for studying the time course of the re-uptake process. We have, therefore, added $^{45}Ca^{2+}$ and carbachol simultaneously to a suspension of isolated acinar cells. Fig. 5.5 shows that carbachol immediately stimulates the $^{45}Ca^{2+}$ uptake, both in the medium with 2.5 mM Ca^{2+} and in that with 0.1 mM Ca^{2+}.

Table 5.3. EFFECT OF STIMULATION BY 10^{-5} M CARBACHOL ON THE $^{45}Ca^{2+}$ CONTENT OF ISOLATED ACINAR CELLS OF RABBIT PANCREAS

Ca^{2+} medium mM	$^{45}Ca^{2+}$-content (% of steady-state content before stimulation)		
	before stimulation	5 min after stimulation	60 min after stimulation
0.1	≙ 100 [1]	47 ± 2	99 ± 8
0.5	≙ 100 [2]	71 ± 2	116 ± 3
2.5	≙ 100 [3]	75 ± 1	151 ± 8

1) 1.0 ± 0.1 nmol $^{45}Ca^{2+}$/mg protein
2) 1.8 ± 0.1 nmol $^{45}Ca^{2+}$/mg protein
3) 3.2 ± 0.3 nmol $^{45}Ca^{2+}$/mg protein

10^{-5} M carbachol is added to the medium after 90 min incubation in the presence of $^{45}Ca^{2+}$.
Mean values with standard error of the mean of four experiments are given.

5.3.8. Effect of extracellular calcium on the stimulation of the enzyme secretion

We have tested whether the effects of extracellular calcium on the uptake of $^{45}Ca^{2+}$ may be related to its effects on the stimulation of the enzyme secretion. For this purpose we have determined the amount of enzyme secreted under the influence of 10^{-5} M carbachol in media with different calcium concentrations. Table 5.4 shows that the magnitude of enzyme secretion depends on the extracellular calcium concentration and that it is significantly higher in media with increasing calcium concentration.

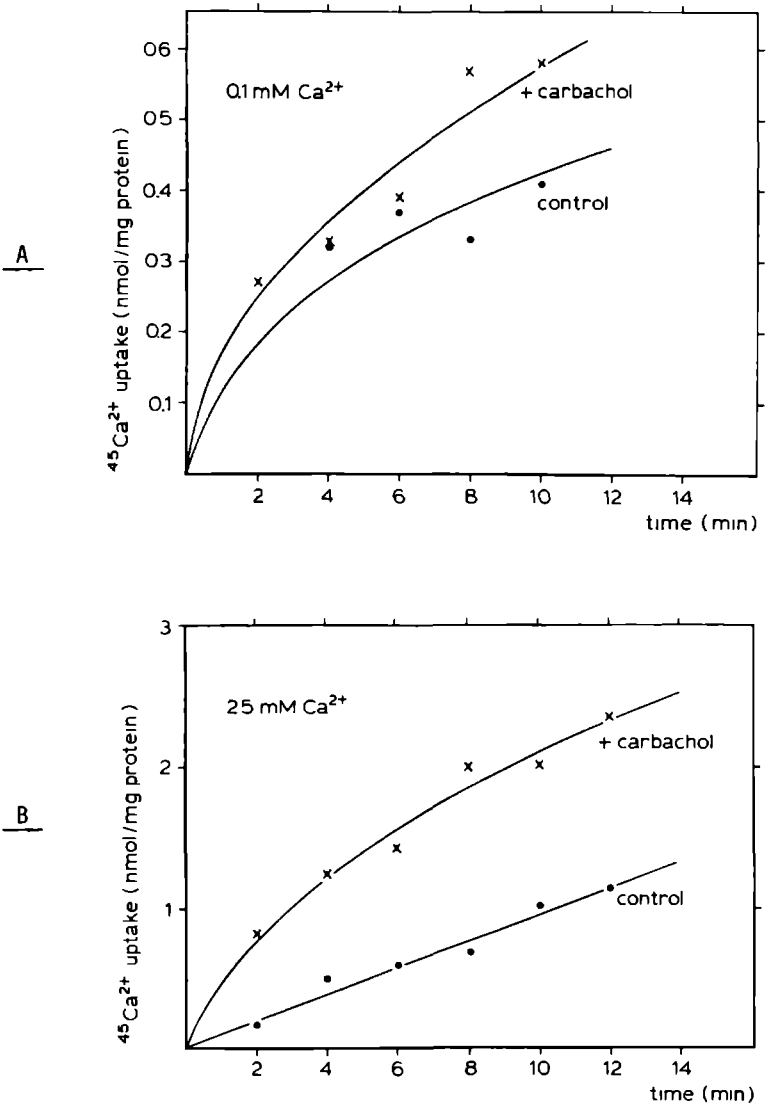


Figure 5.5.

Effect of 10^{-5} M carbachol on $^{45}Ca^{2+}$ uptake by isolated acinar cells. The cells have not been preloaded with $^{45}Ca^{2+}$. At t=0, $^{45}Ca^{2+}$ (control) or $^{45}Ca^{2+}$ plus carbachol are added to the cell suspension.

A. with 0.1 mM extracellular calcium concentration,
B. with 2.5 mM extracellular calcium concentration.
Representative for two (A) and three (B) experiments.

Table 5.4. EFFECT OF MEDIUM CALCIUM CONCENTRATION ON STIMULATION OF THE ENZYME SECRETION

Ca^{2+} in medium mM	Enzyme secretion during 30 min after addition of 10^{-5} M carbachol (secretion without carbachol is taken as 100%)
0	152 ± 13 %
0.1	152 ± 38 %
0.5	200 ± 53 %
2.5	236 ± 40 %

Mean values with standard error of the mean of three experiments are shown. In the calcium-free medium 10^{-4} M EGTA is present. There is a significant correlation between enzyme secretion and calcium level, since the correlation test of Kendall (1948) gives a P-value of 0.035.

5.3.9. Effect of carbachol on release of lactic dehydrogenase

In order to distinguish between stimulation of enzyme secretion and aspecific release of cell proteins, we have measured the release of both amylase and lactic dehydrogenase in a single experiment (Table 5.5). There is no increased release of lactic dehydrogenase after addition of carbachol to either the low calcium or the high calcium medium, while the secretion of amylase is significantly stimulated several fold in both media.

Table 5.5. RELEASE OF LACTIC DEHYDROGENASE AND AMYLASE BEFORE AND AFTER STIMULATION BY CARBACHOL

mM Ca^{2+} in medium	Period	Release of lactic dehydrogenase (% of total per 15 min)	Release of amylase (% of total per 15 min)	nr of determinations
0.1	before stimulation	3.5 ± 0.95	1.3 ± 0.28	4
	after stimulation	4 ± 1.6	3.1 ± 0.66	4
2.5	before stimulation	2.1 ± 0.35	2.9 ± 0.54	2
	after stimulation	2 ± 1.0	10 ± 1.5	4

10^{-5} M carbachol is added to the medium after 90 min incubation. Results are expressed as percent released in 15 min of total activity initially present in cells. Mean values with standard error of the mean are given for the indicated number of determinations.

5.4. Discussion

The study reported in this chapter is aimed at resolving the controversy with regard to the role of calcium in the stimulation of pancreatic enzyme secretion: is the rise in cytoplasmic calcium level in the acinar cells, generally assumed to lead to enzyme secretion, due to an influx of calcium across the basal membrane as suggested by Kondo and Schulz (1976a, b) or to the release of calcium from an intracellular pool as suggested by most other investigators (Case and Clausen, 1973; Schreurs et al., 1975, Gardner et al., 1975; Christophe et al., 1976b).

Calcium movements in isolated acinar cells of the rabbit pancreas have been studied. The advantage of using isolated acinar cells lies in the absence of poorly accessible extracellular spaces around the cells as compared to the situation in intact or sliced pancreas. The rabbit pancreas has been chosen, since the enzyme secretion of its acinar cells is much more sensitive to pancreozymin and carbachol than that of either rat or guinea pig pancreas (Kondo and Schulz, 1976a; Gardner and Jackson, 1977). We achieve a nearly 4-fold stimulation of the enzyme secretion by 10^{-5}M carbachol.

There are certain differences in conditions and procedure between the experiments of Kondo and Schulz and those of Gardner et al., which we have systematically investigated. The former investigators use a lower (0.1%) albumin concentration than the latter (1%). We find that there is no difference in $^{45}Ca^{2+}$ uptake level at these two albumin concentrations. In further experiments we have used 1% albumin, which gives minimal cell lysis. In addition, we have chosen a filtration technique for the determination of ^{45}Ca uptake, and after a comparison of three different washing media and washing techniques we have selected the most advantageous combination.

There is also a difference in calcium concentration in the media used by Kondo and Schulz (1.25 mM) and by Gardner et al. (0.5 mM), which may partly explain why the former investigators find a higher $^{45}Ca^{2+}$ uptake (2.5 nmol/mg protein) than Gardner et al. (0.5 nmol/mg protein, assuming 10^6 cells correspond with 0.5 mg protein).

We do indeed find an increase in the $^{45}Ca^{2+}$ uptake with increasing calcium level in the incubation medium (Table 5.3). However, irrespective of the extracellular calcium concentration used, we find a considerably larger percentage of the cellular calcium (10-20%) exchanging with $^{45}Ca^{2+}$ than both other groups or investigators (5% or less).

The most important difference in procedure is the time of addition of the stimulant: Kondo and Schulz add the stimulant simultaneously with or 30 min before addition of $^{45}Ca^{2+}$, while Gardner et al. add it after preloading the cells with $^{45}Ca^{2+}$. Hence, it is not surprising that the former investigators do not observe the transient decrease in $^{45}Ca^{2+}$ content noticed by Gardner et al. upon addition of the stimulant. We have, therefore, applied both procedures. When we add carbachol to cells preloaded with $^{45}Ca^{2+}$, there is always a transient decrease in the $^{45}Ca^{2+}$ content (Fig. 5.4) in accordance with the findings of Gardner et al. (1975). However, when we add carbachol simultaneously with $^{45}Ca^{2+}$, we observe an increased $^{45}Ca^{2+}$ uptake (Fig. 5.5), which is in accordance with the results of Kondo and Schulz (1976a). In other words, our results show that the divergent observations of these two groups of investigators are primarily due to the difference in time of addition of the stimulant.

Our observations imply that addition of carbachol to the acinar cells causes both a release of calcium from an intracellular pool and an influx of extracellular Ca^{2+} presumably due to an increase in membrane permeability for calcium. The release of calcium from the intracellular pool is probably important for the initiation of the enzyme secretion. This is supported by our observation that even in the absence of extracellular calcium the enzyme secretion can be stimulated (Table 5.4). However, for the continuation of the enzyme secretion replenishment of the calcium pools depleted during the enzyme secretion process appears to be necessary. The increase in membrane permeability for calcium by carbachol facilitates the uptake of calcium required for this purpose. Scheele and Haymovits (1979) also distinguish two components in the secretagogue-induced enzyme release in lobules of the guinea pig pancreas. One component

operates during the initial period after addition of the stimulant and is independent of the extracellular calcium concentration, while the other component depends on the extracellular calcium concentration and operates throughout the stimulation period.

The $^{45}Ca^{2+}$ content of the cells after addition of carbachol reflects the composite effect of stimulation. In the initial period after stimulation $^{45}Ca^{2+}$-efflux predominates, probably due to activation of a plasma membrane Ca^{2+} pump by the increased cytoplasmic calcium concentration. Later $^{45}Ca^{2+}$-influx predominates, leading to restoration of the intracellular calcium pool(s).

The efflux of $^{45}Ca^{2+}$ after addition of carbachol cannot be ascribed to release of calcium sequestered in the zymogen granules. It has been shown (Clemente and Meldolesi, 1975; Schreurs et al., 1976b) that the calcium of the preformed granules does not exchange with $^{45}Ca^{2+}$. Shelby et al. (1976) have provided evidence that upon stimulation there is a release of $^{45}Ca^{2+}$ from a membrane-bound compartment, which slowly equilibrates with the extracellular calcium. Chandler and Williams (1977a, b) have studied the calcium metabolism in acinar cells using the probe chlorotetracycline, which is thought to form a fluorescent complex with membrane associated calcium. They conclude that stimulation of the enzyme secretion causes release of calcium from the mitochondrial pool.

Another difference in the results of Kondo and Schulz and those of Gardner et al. is that the former investigators observed an increased $^{45}Ca^{2+}$ uptake in the presence of a stimulant, while Gardner et al. found a return to the pre-stimulation steady-state level after the transient decrease caused by pancreozymin-octapeptide. Our experiments indicate that this discrepancy is simply due to the different extracellular calcium concentrations used by the two groups (Fig. 5.4 and 5.5). The increased $^{45}Ca^{2+}$ uptake in the presence of a stimulant observed by Kondo and Schulz, cannot be due (at least not entirely) to the use of a choline chloride washing medium, as suggested by Gardner and Hahne (1977), since we find a similar increase with a washing medium without choline chloride.

It remains to be explained why there should occur an increased

$^{45}Ca^{2+}$ uptake after stimulation, at least at a high extracellular calcium level (1.3 - 2.5 mM). Kondo and Schulz (1976a) suggest that it is due to an increased permeability of the membrane for calcium. There is, however, another possible explanation, namely that after stimulation there is a larger replacement of intracellular calcium by $^{45}Ca^{2+}$ than before stimulation. After stimulation there might not only be a replenishment of previously labeled intracellular pools, but also a labeling of a pool which could not be labeled before. The latter pool might represent calcium in the zymogen granules, which does not exchange with $^{45}Ca^{2+}$ when the granules are mature, but which exchange up to 35-50 min after synthesis of the proteins (Schreurs et al., 1979). The latter explanation is supported by our finding that with increasing stimulation of the enzyme secretion more calcium is taken up subsequently (Tables 5.3 and 5.4). The fact that Kondo and Schulz (1976a) measure the same increase in $^{45}Ca^{2+}$ uptake, whether they add the stimulant simultaneously with or 30 min before the addition of $^{45}Ca^{2+}$, indicates that this increased uptake is not involved in stimulus-secretion coupling, but that it is caused by a lingering increase in membrane permeability for calcium or by a reloading of internal calcium store(s).

In conclusion, we feel that our results resolve the discrepancies between the findings of Gardner et al. (1975, 1976) and those of Kondo and Schulz (1976a, b). Our findings and theirs fit the following description of the stimulus-secretion coupling process: the stimulant leads to a release of calcium from an intracellular calcium store, which results in enzyme secretion across the apical membrane and an increased calcium efflux across the basal membrane. In the presence of a high extracellular calcium concentration, the transient efflux of calcium is followed by an increased calcium uptake, which is either due to an increase in membrane permeability for calcium or to a redistribution of intracellular calcium.

The results of our study also provide more information about the calcium pools of the acinar cell. The data in Table 5.3 suggest the presence of at least three calcium pools:

1. a calcium pool (ca 11 nmol/mg protein) which does not exchange with calcium in the medium and which probably represents calcium sequestered in the zymogen granules,
2. a calcium pool, the size of which depends on the extracellular calcium concentration, and which probably represents calcium in cell organelles,
3. a part of pool 2 forms a separate pool 3, which is released upon stimulation. Its localization is not known. Pools 2 and 3 exchange with $^{45}Ca^{2+}$ in the medium.

CHAPTER VI

EFFECT OF SODIUM ON PROTEIN SECRETION AND CALCIUM METABOLISM IN ACINAR CELLS

6.1. Introduction

There are strong indications that the increase in the cytoplasmic calcium concentration, necessary for the initiation of the stimulation of enzyme secretion, is caused by a release of calcium from an intracellular pool by the stimulant (Case and Clausen, 1973; Gardner et al., 1975; Renckens et al., 1978). However, it is not yet sure which calcium pool is involved in this stimulus-secretion coupling. It is possible that calcium bound to the inside of the plasma membrane is the important pool, as is suggested by Petersen and Ueda (1976). Chandler and Williams (1978a, 1978b), however, have provided some evidence that a mitochondrial calcium pool or another pool which requires ATP to sequester calcium is responsible. Regardless of where this calcium pool is located, there must be a coupling between the hormone-receptor interaction and this pool. Neither is it yet sure whether the increase in cytoplasmic calcium concentration stimulates exocytosis directly or via some intermediate steps. So it is conceivable that besides calcium other messengers may play a role in stimulus-secretion coupling.

There are several indications that sodium is involved in pancreatic enzyme secretion. Lowering the extracellular sodium concentration inhibits the stimulation of enzyme secretion in the isolated rat pancreas (Case and Clausen, 1973), mouse pancreatic fragments (Williams, 1975), the perfused rat pancreas (Kanno, 1975; Kanno et al., 1977), rat pancreatic segments (Petersen and Ueda, 1976) and mouse pancreatic acinar cells (Williams et al., 1976). Moreover, Case and Clausen (1973) and Kanno (1975) have observed that incubation in the presence of ouabain reduces the stimulated enzyme secretion. This is, however, in contrast with the finding of

Petersen and Ueda (1977) who do not find any effect of ouabain on amylase release.

Additional evidence for a role of sodium in the stimulus-secretion coupling is provided by electrophysiological experiments. Under normal conditions stimulation of enzyme secretion causes depolarization of the membrane potential(Matthews and Petersen, 1973; Nishiyama and Petersen, 1975; Petersen and Ueda, 1975; Poulsen and Williams, 1977). The fact that this depolarization depends on the extracellular sodium concentration(Matthews and Petersen, 1973; Nishiyama and Petersen, 1975) leads to the conclusion that the stimulant causes an increase in the membrane permeability for sodium and consequently a Na^+-influx (Nishiyama and Petersen, 1975; Petersen and Ueda, 1976).

The above data suggest that sodium is indeed involved in pancreatic enzyme secretion. We have investigated in which step(s) of the stimulus-secretion coupling process sodium interferes, particularly with respect to the relation between calcium and sodium transport in the acinar cell. We have investigated in this chapter whether lowering the extracellular sodium concentration and addition of ouabain have an effect on the stimulation of enzyme secretion and calcium metabolism of the acinar cells. We discuss which step(s) in the stimulus-secretion coupling process may be influenced by changes in sodium metabolism.

6.2. Materials and methods

6.2.1. Materials

$^{45}CaCl_2$ (800 Ci/mol), ^{3}H-leucine (1 Ci/mmol) and ^{3}H-inulin (900 Ci/mmol) are purchased from the Radiochemical Centre (Amersham, U.K.) and ^{14}C-tetraethylammonium bromide (4.4 Ci/mmol) from New England Nuclear (Boston, Mass. U.S.A.). Hyamine hydroxide 10-X, Picofluor TM and Instagel are obtained from Packard Instrument s.a. (Brussels, Belgium), the Phadebas amylase test from Pharmacia (Uppsala, Sweden) and A23187 is a gift from the Eli Lilly Company (Indianapolis, U.S.A.).

6.2.2. Tissue preparation

Isolated acinar cells of rabbit pancreas are prepared as described in section 5.2.2.

Pancreatic fragments are prepared by cutting the pancreas in pieces of about 250 mg wet weight.

6.2.3. Incubation medium

The pancreatic fragments are incubated in a Krebs-Ringer bicarbonate medium, the composition of which is given in section 2.2.4. In the case of isolated acinar cells trypsin inhibitor (0.2 mg/ml) and bovine serum albumin (1% w/v) are added to this medium. During (pre)incubation the medium is constantly gassed with an O_2/CO_2 mixture to keep the pH at 7.4. When a low sodium medium is used, the omitted sodium chloride is replaced by an isosmotic concentration of tetraethylammonium chloride or sucrose as indicated in the text.

6.2.4. Protein secretion

Enzyme secretion from acinar cells is determined by measuring the release of trichloroacetic acid-insoluble ^3H-labeled proteins as described in section 5.2.4.

When amylase secretion from pancreatic fragments is measured, the fragments are preincubated for 30 min and then one each is transferred to a vial containing 5 ml fresh medium. After fixed time periods the fragments are transported to fresh medium containing the agents indicated in the text. At the end of incubation the fragments are homogenized in a Potter-Elvehjem tube. Amylase activity is measured in each vial and in the homogenate, and the amylase release is expressed as percent of total amylase activity present in the fragment at the start of incubation.

6.2.5. Determination of ion contents of isolated acinar cells

To measure the content of a certain ion in acinar cells we centrifuge an aliquot of the cell suspension, and measure the content of that ion in the total pellet. By correcting this value for the

ion content in the inulin space the cellular content of that ion is calculated and expressed as nmol/mg protein. The exact procedure has been described for calcium in section 5.2.6. However, the procedure has been modified by adding 10 mM EDTA before centrifugation of the sample in order to remove calcium adhering to the outside of the cells. The $^{45}Ca^{2+}$-content is measured in the same way after the cells are incubated for 90 min in Krebs-Ringer bicarbonate medium containing 2.5 µCi/ml $^{45}Ca^{2+}$. The radioactivity in the samples is counted after addition of 4 ml Picofluor TM.

To check whether the cells take up tetraethylammonium ions, they are incubated in a medium containing 25 mM Na^+ and 118.5 mM tetraethylammonium chloride, to which is added 0.2 µCi/ml ^{14}C-tetraethylammonium bromide. By measuring the radioactivity in the samples, the amount of tetraethylammonium in the cell can be calculated after correction for the isotope in the inulin space.

The sodium and potassium contents of the samples are determined by means of flame photometry.

6.2.6. Efflux of $^{45}Ca^{2+}$

The $^{45}Ca^{2+}$-efflux from acinar cells is measured according to the method of Peikin et al. (1979). The cells of one pancreas are suspended in 1 ml Krebs-Ringer bicarbonate medium containing 2.5 µCi $^{45}Ca^{2+}$ and are incubated for 90 min at 37 °C. Then the cells are washed twice and are resuspended in 6 ml medium. One-ml aliquots of the suspension are incubated for 5 min with or without carbachol. At the beginning and end of the incubation duplicate 175-µl samples are centrifuged (5 sec, minicentrifuge) through 200 µl maize oil/phthalate mixture (d=1.02) and the radioactivity in 100 µl of the supernatant is counted. The efflux is expressed as percent of total radioactivity in the cells.

The $^{45}Ca^{2+}$-efflux from pancreatic fragments is determined by the method described by Schreurs et al. (1975): the pancreas is loaded with $^{45}Ca^{2+}$ by incubation for 2 hours in about 1.5 ml Krebs-Ringer bicarbonate medium containing 10 µCi $^{45}Ca^{2+}$ and is then cut in fragments, which are washed for 15 min in excess medium to

remove adhering radioactivity. At fixed time intervals the fragments are transferred to a vial containing 5 ml fresh medium, and the radioactivity in each vial is counted after addition of 10 ml Instagel. The radioactivity left in the fragment is determined after destruction in Hyamine hydroxide 10-X. The efflux rate of each fraction is calculated as

$$k(min^{-1}) = \frac{dpm.min^{-1} \text{ in sample}}{\text{mean dpm in tissue}}$$

6.2.7. Assay methods

Protein concentration is measured according to Lowry et al. (1951), using bovine serum albumin as a standard.

Amylase activity is measured in appropriate dilutions of the samples by means of the Phadebas test, modified for assay on a microscale. Since some agents present in the samples, like ouabain, tetraethylammonium and A23187, appear to interfere with the assay reaction, the results are corrected by using standards containing these agents.

Radioactivity is counted in a Philips liquid scintillation analyzer. In double-labeling experiments the radioactivity of each isotope is calculated by means of the external standard ratio method.

6.3. Results

6.3.1. Effect of extracellular Na^+ concentration and ouabain on enzyme secretion by isolated acinar cells.

To check whether sodium is involved in the enzyme secretion by the rabbit pancreas, isolated acinar cells are incubated in Krebs-Ringer bicarbonate medium containing 25 mM Na^+ (the rest of the sodium being replaced by tetraethylammonium) and in Krebs-Ringer bicarbonate medium containing 10^{-5}M ouabain. Fig.6.1 shows the effects on the basal and the carbachol-induced enzyme release. Incubation in a medium with 25 mM Na^+ causes a small, not significant rise in

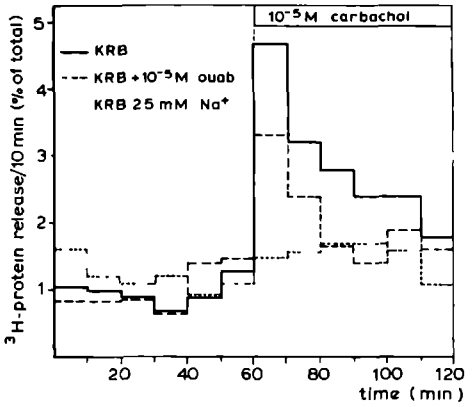


Figure 6.1.

Effect of 10^{-5}M carbachol on release of ^{3}H-protein from isolated acinar cells in normal KRB medium, KRB medium containing 25 mM Na^{+} (sodium being replaced by tetraethylammonium) and KRB medium containing 10^{-5}M ouabain. Means of 8, 3 and 5 experiments, respectively.

basal enzyme release, which may be due to an increased breakdown of the cells in this medium. The carbachol-induced enzyme secretion is largely inhibited by the low extracellular sodium concentration. Ouabain has no effect on the basal enzyme release and blocks the stimulation by carbachol only partly. In Table 6.1 values are given for the enzyme release during the initial 20-min period and during the 60-min period following addition of carbachol to the various media. In normal Krebs-Ringer bicarbonate medium carbachol causes an initial 4-fold stimulation of the enzyme secretion, which slowly levels off. In a medium containing 25 mM Na^{+} the enzyme release is constant during the 60-min period following addition of carbachol and is only slightly higher than the basal secretion rate. In a medium containing ouabain there is a peak in the enzyme release after

Table 6.1. EFFECT OF EXTRACELLULAR SODIUM CONCENTRATION AND OUABAIN ON ENZYME SECRETION

	Basal release	Release initial 20 min after addition of carb.	Release 60 min period after addition of carb.
KRB	2.0±0.3(10)	8.8±0.9(10)	5.8±0.6(8)
KRB, 25 mM Na^+	2.4±0.2(3)	3.1±0.4(3)	3.1±0.2(3)
KRB, 10^{-5}M ouabain	2.1±0.3(7)	5.8±0.8(7)	4.1±0.4(5)
KRB, 25 mM Na^+ 10^{-5}M ouabain	1.9±0.2(3)	3.9±0.7(3)	1.9±0.5(3)

Carbachol is added after 60 min incubation in the various media. Enzyme release is expressed as percent of total enzyme content released per 20 min. In the 25 mM Na^+ media sodium is replaced by tetraethylammonium. Mean values with standard error of the mean are given, with the number of determinations (n) in parentheses.

addition of carbachol, but this peak is smaller than the one in normal Krebs-Ringer bicarbonate medium. When ouabain is added in the 25 mM Na^+ medium, the effect is slightly higher than in normal sodium medium.

6.3.2. Effect of ouabain concentration on carbachol-induced enzyme release

Fig.6.1 shows that incubation in a medium containing 10^{-5}M ouabain inhibits the carbachol-induced enzyme release only partly. To check whether the stimulation can be completely blocked by higher ouabain concentrations, isolated acinar cells are incubated for 60 min in media containing various ouabain concentrations and the amount of enzyme released during a 20 min period following carbachol addition is measured. Fig.6.2 shows that the maximal inhibitory effect is reached at about 3.10^{-6}M ouabain and that even at the highest ouabain concentration tested (3.10^{-4}M) carbachol still causes an

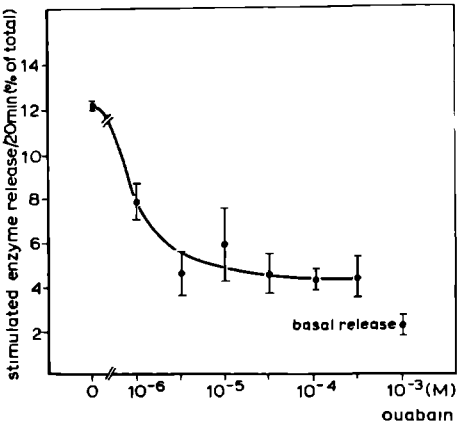


Figure 6.2.

Effect of ouabain concentration on the carbachol-induced enzyme release from isolated acinar cells. The cells are incubated 60 min in the presence of ouabain before the addition of carbachol. Values are percentages of ^{3}H-protein released in a 20 min period after addition of 10^{-5}M carbachol. Means with standard error of the mean for two experiments.

about 2-fold stimulation of enzyme secretion. In all further experiments 10^{-4}M ouabain is used.

6.3.3. Effect of extracellular Na^{+} concentration and ouabain on the cellular sodium and potassium content

In order to see whether the sodium metabolism of the pancreas is indeed affected by incubation in a medium with low Na^{+} concentration or with ouabain, the cellular contents of sodium and potassium are measured following 60 min incubation in a medium with 25 mM Na^{+}, in a medium with 10^{-4}M ouabain and in a medium containing both 25 mM Na^{+} and 10^{-4}M ouabain. The resulting values are compared

Table 6.2. *EFFECT OF EXTRACELLULAR SODIUM CONCENTRATION AND OUABAIN ON THE CELLULAR SODIUM AND POTASSIUM CONTENTS*

	Na^+ content (nmol/mg prot.)	K^+ content (nmol/mg prot.)	Sum Na^+ and K^+ contents	n
KRB	174 ± 42*	528 ± 23*	702 ± 48	4
KRB, 25 mM Na^+	101 ± 24	396 ± 58	497 ± 93	4
KRB, 10^{-4}M ouabain	596 ± 37	164 ± 20	760 ± 42	3
KRB, 25 mM Na^+ 10^{-4}M ouabain	234 ± 68	194 ± 24	428 ± 72	3

The Na^+ and K^+ contents are measured after 60 min incubation in the indicated media. In the 25 mM Na^+ media sodium is replaced by tetraethylammonium. Mean values are given with standard error of the mean (n is the number of experiments).

* *The values for the sodium and potassium contents correspond with 35 and 120 mM, respectively, assuming that 50% of the wet weight of the tissue is intracellular fluid and 11% of the wet weight is protein.*

with those obtained after incubation in normal Krebs-Ringer bicarbonate medium (Table 6.2).

Under normal conditions the potassium content is about 120 mM and the sodium content about 35 mM. As expected, incubation in the presence of ouabain causes an increase in the sodium content and a decrease in the potassium content of the cells, the sum of both ion concentrations remaining constant. However, incubation in the low sodium medium with or without ouabain reduces both the sodium and potassium contents of the cells. This indicates that another cation must be present in the cells to maintain isotonicity, presumably tetraethylammonium ion, which is used to replace sodium. Experiments with radioactive tetraethylammonium show that after 60 min incubation in a medium with 25 mM Na^+ (containing 118 mM tetraethylammonium) 199 nmol/mg protein tetraethylammonium is present in the cells (mean of two experiments). The sum of the contents of potassium, sodium and tetraethylammonium is in that case 696 nmol/mg protein, which is equal to the sum of the sodium and potassium contents after incubation in normal Krebs-Ringer bicarbonate medium. So apparently tetraethylammonium can penetrate and partly replace sodium and potassium.

6.3.4. Effect of extracellular sodium concentration and ouabain on the exchangeable and total cellular calcium contents

The $^{45}Ca^{2+}$ content and the total calcium content of the cells is measured after 90 min incubation in normal Krebs-Ringer bicarbonate medium, in medium containing 25 mM Na^+, in medium containing 10^{-4}M ouabain and in medium containing both 25 mM Na^+ and 10^{-4}M ouabain. The calcium concentration in all four media is 2.5 mM. The results are shown in Table 6.3. The total cellular calcium content after incubation in normal medium is somewhat lower than previously found (see section 5.3.5), but this is probably due to the modification of the procedure as described in section 6.2.5 (addition of 10 mM EDTA before centrifugation). Lowering the sodium concentration or adding ouabain to the incubation medium seems to have no effect

Table 6.3. EFFECT OF EXTRACELLULAR SODIUM CONCENTRATION AND OUABAIN ON THE EXCHANGEABLE AND TOTAL CELLULAR CALCIUM CONTENTS

	Exchangeable Ca content (nmol/mg prot)	Total Ca content (nmol/mg prot)
KRB	3.9 ± 0.7 (14)	12.2 ± 0.9 (21)
KRB, 25 mM Na^+	6.9 ± 1.2 (10)	12.4 ± 2.1 (13)
KRB, 10^{-4}M ouabain	7.0 ± 1.8 (5)	12.4 ± 1.7 (5)
KRB, 25 mM Na^+ 10^{-4}M ouabain	9.3 ± 1.1 (5)	14.5 ± 1.3 (5)

Calcium contents are measured after 90 min incubation in the various media. In the 25 mM Na^+ media sodium is replaced by tetraethylammonium. Mean values with standard error of the mean are given, with the number of determinations (n) in parentheses.

on the total calcium content of the cells, while the $^{45}Ca^{2+}$ content of the cells increases under these conditions. This increase is further enhanced by incubation in a medium containing both 25 mM Na^+ and 10^{-4}M ouabain, which also causes a small rise of the total cellular calcium content.

6.3.5. Effect of extracellular sodium concentration and ouabain on the carbachol-induced $^{45}Ca^{2+}$-efflux from isolated acinar cells.

We have checked whether incubation in medium with low sodium concentration or with ouabain has an effect on the Ca^{2+}-efflux under the influence of carbachol. Isolated acinar cells are incubated for 90 min in the various media containing $^{45}Ca^{2+}$, extracellular $^{45}Ca^{2+}$ is washed away and the amount of $^{45}Ca^{2+}$ released to the medium in a 5 min period is measured. In normal Krebs-Ringer bicarbonate medium carbachol causes a 4-fold increase of the $^{45}Ca^{2+}$-efflux (Table 6.4). When the cells are incubated in a medium containing 25 mM Na^+, with

Table 6.4. EFFECT OF EXTRACELLULAR SODIUM CONCENTRATION AND OUABAIN ON THE CARBACHOL INDUCED $^{45}Ca^{2+}$-EFFLUX

	$^{45}Ca^{2+}$-efflux	
	Control	10^{-5}M carbachol
KRB	6 ± 1.1	23 ± 7.7
KRB, 25 mM Na^+	5 ± 2.6	5 ± 3.0
KRB, 10^{-4}M ouabain	4.8 ± 0.4	11 ± 2.2
KRB, 25 mM Na^+ 10^{-4}M ouabain	5 ± 2.4	5 ± 2.2

Values are the amount of $^{45}Ca^{2+}$ appearing in the medium in a 5 min period, expressed as percent of total cellular $^{45}Ca^{2+}$ content. Carbachol is added after 90 min incubation in the indicated media. In the 25 mM Na^+ medium sodium is replaced by tetraethylammonium. Mean values with standard error of the mean of three separate experiments.

or without ouabain, there is no influence of carbachol on the $^{45}Ca^{2+}$-efflux. On the other hand, carbachol is able to cause an increased $^{45}Ca^{2+}$-efflux from cells incubated in normal Krebs-Ringer bicarbonate medium containing ouabain.

6.3.6. Short-term effects of ouabain on enzyme secretion and calcium metabolism in pancreatic fragments

In all experiments described so far the effect of carbachol is studied after the cells have been incubated for at least 60 min in the various media. This means that at the time carbachol is added both the intracellular ionic concentrations and the ion fluxes across the plasma membrane have been changed. To determine which of these two phenomena is responsible for the observed effects, experiments

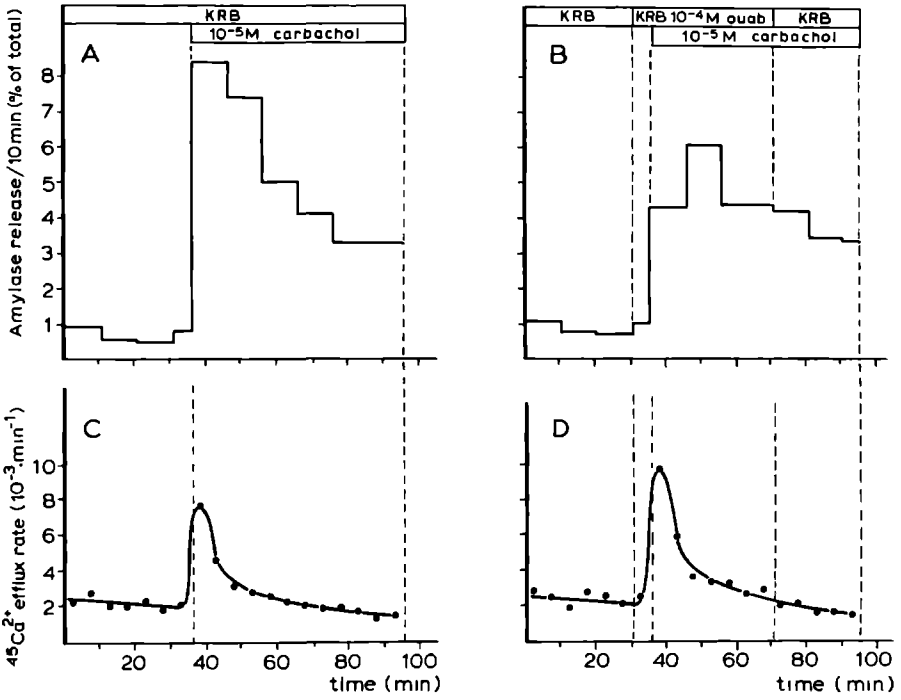


Figure 6.3.

Effect of 10^{-5}M carbachol on the amylase release and $^{45}Ca^{2+}$-efflux from pancreatic fragments in normal KRB medium (A,C) and in KRB medium containing 10^{-4}M ouabain (B,D). The efflux during the first 90 min period after removing extracellular $^{45}Ca^{2+}$ is not shown. Means of 4 (A,B), 5 (C) and 3 (D) experiments.

are done in which the Krebs-Ringer bicarbonate medium is replaced by the other media only 5 min before the addition of carbachol. It is unlikely that the cellular ion concentrations are then changed very much at the time carbachol is added, so that primarily the ion fluxes

across the membrane are influenced. In these experiments pancreatic fragments are used, because they permit a quick change of the medium. Comparison of Figs. 6.1 and 6.3a shows that in normal Krebs-Ringer bicarbonate medium the pattern of enzyme release from pancreatic fragments and from isolated acinar cells is similar. Only the stimulation of enzyme secretion is somewhat higher in pancreatic fragments than in isolated acinar cells. This is probably due to partial damage of the receptors during isolation of the cells. Based on the above observations we have concluded that the results of experiments with pancreatic fragments and with isolated cells are comparable.

Figs. 6.3a and 6.3b show the short-term effect of ouabain on the carbachol-induced enzyme secretion. In the presence of ouabain the amount of enzyme released in the first 20 min following addition of carbachol (9.6%, S.E. 2.3, n=4) is significantly ($p < 0.05$ for paired values) lower than in normal Krebs-Ringer bicarbonate medium (15.9%, S.E. 2.7, n=4). Removal of ouabain does not cause an additional peak of enzyme release, which suggests that the effect of ouabain is not reversible. However, this may also be caused by the fact that ouabain remains bound to the cells even when it is removed from the medium. From Figs. 6.3c and 6.3d it is obvious that the ability of carbachol to increase the $^{45}Ca^{2+}$-efflux is not inhibited by the presence of ouabain. These results are analogous to those found for isolated acinar cells after prolonged incubation with ouabain.

6.3.7. Short-term effects of low extracellular sodium concentration on enzyme secretion and calcium metabolism in pancreatic fragments

When the sodium concentration of the medium is lowered to 25 mM (by isotonic replacement with tetraethylammonium) 5 min before addition of carbachol, the carbachol-induced enzyme secretion is nearly completely abolished (Fig. 6.4a). When the low sodium medium is then replaced by normal Krebs-Ringer bicarbonate medium containing carbachol, there is a marked increase in enzyme release. This indicates that the effect of low sodium is reversible, although a

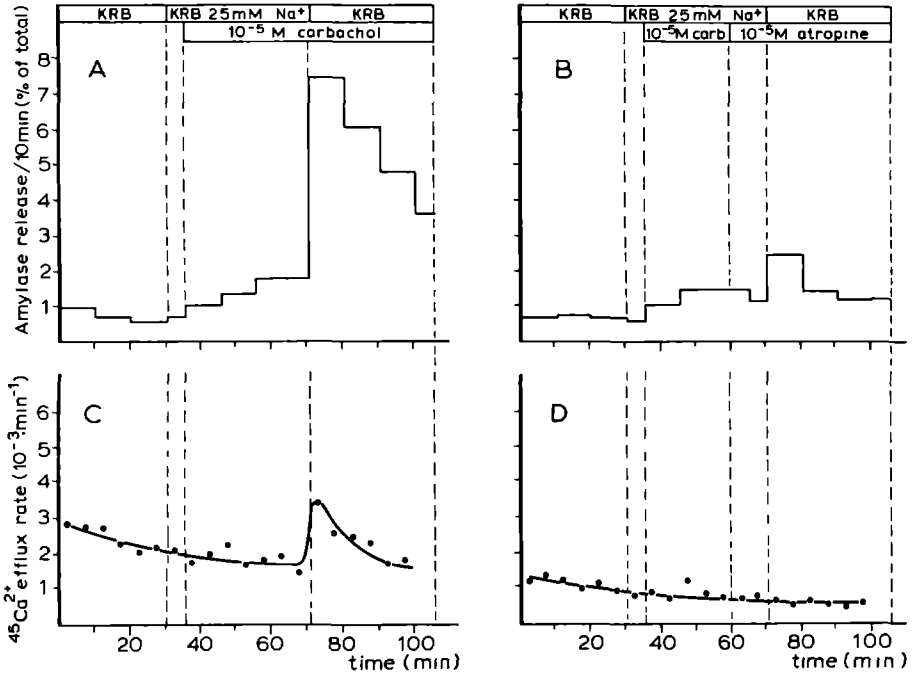


Figure 6.4.

Effect of 10^{-5}M carbachol on amylase release and $^{45}Ca^{2+}$-efflux from pancreatic fragments in KRB medium containing 25 mM Na^{+} (sodium being replaced by tetraethylammonium) and effect of replacing this medium again by normal KRB medium, carbachol still being present (A,C) or carbachol being replaced by atropine (B,D). The efflux during the first 90 min after removing extracellular $^{45}Ca^{2+}$ is not shown. Means of 5 (A,C), 3 (B) and 2 (D) experiments.

wash-out effect due to inhibition of fluid secretion in low sodium medium cannot be completely excluded because of the presence of the

ductular system in the pancreatic fragments. The latter suggestion is not very likely, since stimulation of the enzyme secretion is also inhibited when isolated acinar cells are incubated in a medium with low sodium concentration (Fig. 6.1). We have checked this possibility by replacing carbachol with atropine 10 min before changing to normal medium. In that case we do not find additional release of enzyme, while the fluid secretion, which is reduced in the low sodium medium, is restored to normal values under that condition (Petersen and Ueda, 1977). This indicates that the inhibition of the enzyme secretion is not due to inhibition of the fluid secretion.

From Figs. 6.4c and 6.4d it is obvious that carbachol does not cause an increased $^{45}Ca^{2+}$-efflux, when the sodium concentration in the medium is lowered to 25 mM 5 min before addition of carbachol. Replacing the medium again by normal Krebs-Ringer bicarbonate medium results in a slight increase in $^{45}Ca^{2+}$-efflux when carbachol is present. Prolonged incubation in low sodium medium before addition of carbachol gives the same results as shown in Fig. 6.4.

These results can be explained in two ways: 1) carbachol is unable to release calcium from the intracellular pool when the extracellular sodium concentration is 25 mM; 2) carbachol causes a release of calcium from the intracellular pool, but under this condition the calcium cannot leave the cell. The latter possibility has been investigated by using the calcium ionophore A23187. This substance causes in 10^{-6}M concentration an increase in the membrane permeability for calcium in pancreatic fragments (Schreurs et al., 1976a) without other drastic effects on the metabolism of the cells (Williams, 1978). Fig. 6.5 shows an experiment in which low sodium medium containing A23187 is added to a pancreatic fragment. Calcium-free medium is used in order to prevent calcium influx, but this has under normal conditions no effect on the $^{45}Ca^{2+}$-efflux pattern. It is clear that in the presence of A23187 carbachol also causes no increase in the $^{45}Ca^{2+}$-efflux in low sodium medium. This implies that under this condition there is no increase in cytoplasmic calcium concentration by carbachol. So apparently in the low sodium medium the coupling between hormone-receptor interaction and release

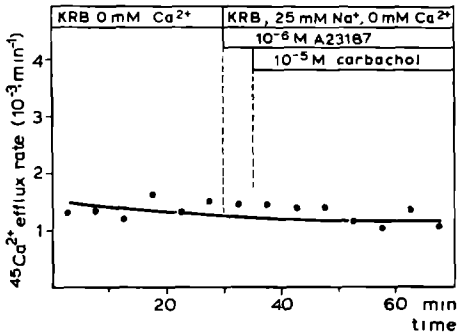


Figure 6.5.

Effect of 10^{-5}M carbachol on $^{45}Ca^{2+}$-efflux in calcium-free KRB medium containing 25 mM Na^{+} and 10^{-6}M A23187 (sodium being replaced by tetraethylammonium). The efflux during the first 90 min after removing extracellular $^{45}Ca^{2+}$ is not shown. Means of 4 experiments.

of calcium from the intracellular pool is blocked.

Raising the cytoplasmic calcium concentration by incubation in the presence of A23187 and calcium results in stimulation of the enzyme secretion (Fig. 6.6a). However, when the extracellular sodium concentration is lowered to 25 mM, stimulation is considerably inhibited (Fig. 6.6b). This inhibition is reversible, since replacement of the low sodium medium by normal Krebs-Ringer bicarbonate medium results in a peak of enzyme release. This indicates that in the low sodium medium the coupling between intracellular calcium release and exocytosis is blocked.

6.3.8. Effects of replacing sodium chloride by sucrose on enzyme secretion and calcium metabolism in pancreatic fragments

In all above experiments in which a low sodium medium is used, the omitted sodium is replaced isotonically by tetraethylammonium.

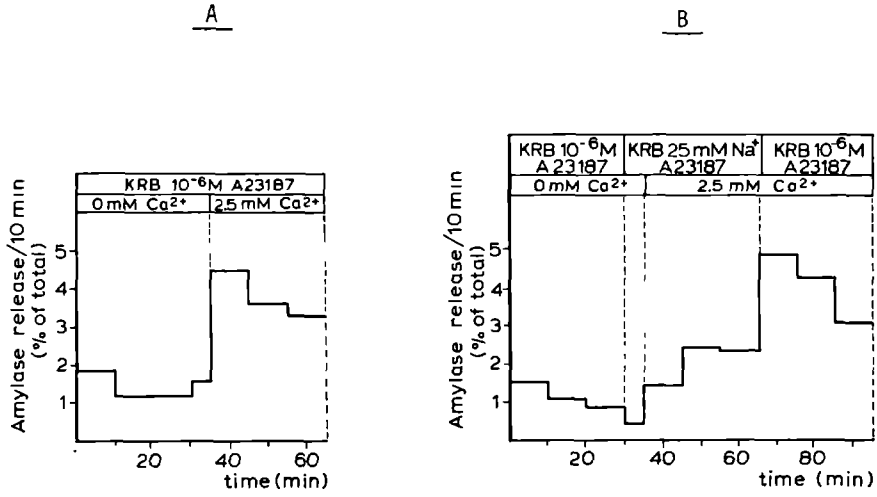


Figure 6.6.

Effect of 10^{-6}M A23187 in the presence of calcium on amylase release from pancreatic fragments in normal KRB medium (A) and in 25 mM Na^+ medium (B, sodium being replaced by tetraethylammonium). Means of 4 experiments.

However, since this substance seems to penetrate the cell (see 6.3. 3), it may cause additional effects. Therefore, we have also used sucrose to replace the omitted sodium chloride, although this has the disadvantage that not only the sodium but also the chloride concentration is lowered.

Incubation in the sucrose medium with 25 mM Na^+, with or without preincubation in this medium, shows a reversible inhibition of the carbachol-induced enzyme secretion (Figs. 6.7a and 6.7b), just as we have found when tetraethylammonium is used to replace sodium. On the other hand, the effect on the $^{45}Ca^{2+}$-efflux pattern is somewhat different. When the Krebs-Ringer bicarbonate medium is replaced

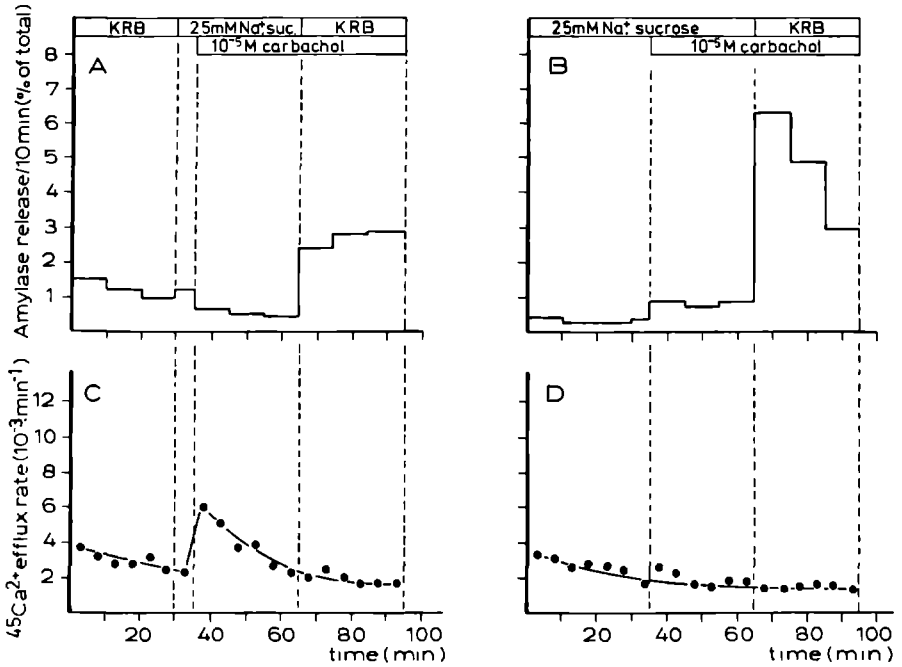


Figure 6.7.

Effect of 10^{-5}M carbachol on amylase release and $^{45}Ca^{2+}$-efflux from pancreatic fragments in 25 mM Na^{+} medium (sodium chloride being replaced by sucrose) after 5 min (A,C) and after prolonged (B,D) preincubation in this medium. The efflux during the first 90 min after removing extracellular $^{45}Ca^{2+}$ is not shown. Means of 3 (A,C,D) and 2(B) experiments.

by the sucrose medium 5 min before addition of carbachol, this stimulant causes an increased $^{45}Ca^{2+}$-efflux, which does not occur when sodium is replaced by tetraethylammonium (Fig. 6.4c). This means

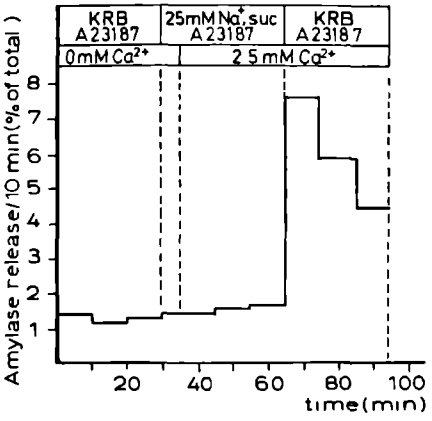


Figure 6.8.

Effect of 10^{-6}M A23187 in the presence of calcium on amylase release from pancreatic fragments in 25 mM Na^+ medium (sodium chloride being replaced by sucrose). Mean of 3 experiments.

that in the sucrose medium the inhibition of the carbachol-induced enzyme secretion is caused by an interruption of the stimulus-secretion coupling after the rise in cytoplasmic calcium concentration. After prolonged incubation (120 min) in the sucrose medium the carbachol-induced $^{45}Ca^{2+}$-efflux disappears (Fig. 6.7d), indicating that now also the coupling between hormone-receptor interaction and calcium release is blocked. Replacing the medium again by normal Krebs-Ringer bicarbonate medium does not result in an increased $^{45}Ca^{2+}$-efflux, as might have been expected from the enzyme secretion pattern.

The experiment shown in Fig.6.8, in which A23187 is used to increase the cytoplasmic calcium concentration, confirms that in the sucrose medium, just as in the tetraethylammonium medium, a step between calcium release and exocytosis is blocked.

6.4. Discussion

The aim of this study has been to determine which role sodium ions play in pancreatic enzyme secretion. It was already known that lowering the extracellular sodium concentration inhibits stimulation of enzyme secretion in the isolated rat pancreas (Case and Clausen, 1973), in the perfused rat pancreas (Kanno, 1975; Kanno et al., 1977; Petersen and Ueda, 1977), in rat pancreatic segments (Petersen and Ueda, 1976) and in mouse pancreatic fragments (Williams, 1975). The observation that stimulation of enzyme secretion from isolated mouse pancreas acinar cells is only inhibited after prolonged preincubation in Na^+-free medium (Na^+ being replaced by $Tris^+$), led Williams et al. (1976) to the conclusion that inhibition of stimulated enzyme secretion by mouse pancreatic fragments in Na^+-free medium is due to inhibition of the fluid secretion. The same explanation is given by Petersen and Ueda (1977) for the inhibition of the acetylcholine-induced enzyme secretion in Na^+-free medium (Na^+ being replaced by $Tris^+$) in the perfused rat pancreas.

In this study the stimulation of the enzyme secretion by carbachol is measured in isolated acinar cells of rabbit pancreas after 60 min preincubation in a medium containing 25 mM Na^+, and in rabbit pancreatic fragments immediately after lowering the extracellular sodium concentration. In the experiments with isolated cells tetraethylammonium is used to replace sodium, while in the experiments with fragments both tetraethylammonium and sucrose are used to replace sodium. In all cases the stimulation of the enzyme secretion by carbachol is inhibited, and for pancreatic fragments this inhibition is reversible. Fig.6.4b shows that if carbachol is replaced by atropine just before addition of normal Krebs-Ringer bicarbonate medium, there is no peak of enzyme release. This means that the peak measured in Fig.6.4a is not caused by a wash-out effect due to inhibition of the fluid secretion in the low sodium medium. The inhibition of the carbachol-induced enzyme release in low sodium medium might be caused by a decreased affinity of the receptor for carbachol under this condition. However, Birdsall et al. (1979) have shown that

in a synaptosomal preparation sodium ions do not affect the binding of carbachol to the muscarinic receptor. So this possibility is not very likely.

Ouabain also inhibits stimulation of the enzyme secretion by carbachol in isolated acinar cells and pancreatic fragments, both with and without preincubation, although the inhibitory effect is less pronounced than in the low sodium medium. Case and Clausen (1973) find in the isolated rat pancreas a 60% inhibition of the stimulated enzyme secretion after 85 min preincubation in the presence of ouabain, while Kanno (1975) observes an 80% inhibition of the pancreozymin-induced enzyme release in rat pancreas by ouabain after 30 min preincubation in the presence of ouabain. On the other hand, Petersen and Ueda (1977) find, without preincubation in the presence of ouabain, no inhibitory effect of ouabain on the caerulein-induced enzyme release in the perfused rat pancreas. This observation is in disagreement with our finding in rabbit pancreatic fragments, and may be due to the lower sensitivity of rat tissue for ouabain (Bonting, 1970, p 267). The fact that the inhibition of the carbachol-induced enzyme release also occurs after only 5 min preincubation in a medium containing 25 mM Na^+ or ouabain indicates that not the intracellular ionic concentrations, but rather the active ion fluxes across the membrane are responsible for the effect.

Because it is known that Ca^{2+} ions play an important role in pancreatic enzyme secretion, we have determined the effects of lowering the extracellular sodium concentration or addition of ouabain on the distribution and transport of calcium in pancreatic acinar cells. Table 6.3 shows that incubation in medium containing 25 mM Na^+ and in medium containing ouabain causes an increase in the $^{45}Ca^{2+}$-content of the cells. When the sodium concentration is lowered to 25 mM and also ouabain is added to the medium, this increase is further enhanced. The increased $^{45}Ca^{2+}$-content observed in the presence of 25 mM Na^+ is in agreement with the two-fold increase in $^{45}Ca^{2+}$-uptake in isolated acinar cells of rat pancreas observed by Schulz et al. (1977) under that condition. This phenomenon may be explained by the operation of a Na-Ca-exchange mechanism in the plasma membrane.

When the sodium gradient across the membrane is removed, by lowering the extracellular sodium concentration or by incubation in the presence of ouabain, the sodium influx decreases and consequently the Ca^{2+}-efflux is also reduced, resulting in a rise of the cellular calcium content. The same phenomenon is observed in smooth muscle (Casteels and Van Breemen, 1975; Ma and Bose, 1977). However, this hypothesis would imply that the total calcium content of the cells would have to increase by about the same amount as the exchangeable calcium content. Table 6.3 shows that we find no indication for such an increase, except when a combination of low sodium and ouabain is used. But in that case there is also an additional increase in the exchangeable calcium content.

The fact that the total cellular calcium content does not seem to increase in medium conteining 25 mM Na^+ or ouabain, suggests that the increase in $^{45}Ca^{2+}$-content may be due to the labeling of a calcium pool, which under normal conditions does not exchange with $^{45}Ca^{2+}$. It is not yet clear which calcium pool might be involved, but a possible candidate is the calcium sequestered in the zymogen granules, for it is shown that this pool does not exchange with $^{45}Ca^{2+}$ under normal conditions (Schreurs et al., 1979). This point deserves further investigation. However, since lowering of the extracellular sodium concentration or addition of ouabain to the medium immediately affects the stimulated enzyme secretion, it is not very likely that a change in the calcium distribution in the cells is responsible for the inhibitory effect.

It was already known that addition of carbachol to pancreatic fragments, preloaded with $^{45}Ca^{2+}$, increases the $^{45}Ca^{2+}$-efflux rate (Case and Clausen, 1973; Matthews et al., 1973; Schreurs et al., 1975). A similar phenomenon is observed in dispersed acini (Peikin et al., 1979). This efflux is thought to be the result of a release of calcium from an intracellular pool under the influence of a stimulant. We have checked whether this $^{45}Ca^{2+}$-efflux is affected by incubation in a medium containing 25 mM Na^+ or ouabain. From Figs.6.3c and d and Table 6.4 it is clear that in the presence of ouabain carbachol also causes an increased $^{45}Ca^{2+}$-efflux, in agreement with

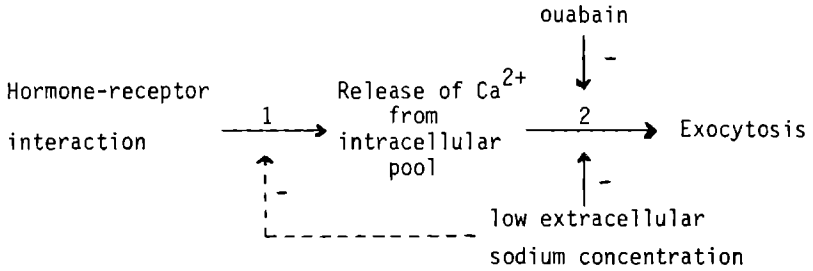


Figure 6.3.

Schematic presentation of the pancreatic stimulus-secretion coupling.

earlier findings of Case and Clausen (1973). This means that ouabain does not prevent the release of calcium from the intracellular pool by carbachol, but that the inhibitory effect takes place in a later phase of the stimulus-secretion coupling (see Fig.6.9). It is too early to speculate about the step, which is blocked by the inhibition of Na^+-K^+ATPase.

The effect of a low sodium medium on the carbachol-induced $^{45}Ca^{2+}$-efflux seems to depend on the substance which is used to replace the omitted sodium. When tetraethylammonium is used, carbachol is unable to cause an increased $^{45}Ca^{2+}$-efflux from isolated cells and pancreatic fragments, even after only 5 min preincubation in the low sodium medium (Table 6.4 and Fig.6.4c). Fig.6.5 shows that also in the presence of A23187 carbachol does not cause an increased $^{45}Ca^{2+}$-efflux in the tetraethylammonium medium, which implies that in this medium carbachol is unable to release calcium from the intracellular pool. In the sucrose low sodium medium the carbachol-induced $^{45}Ca^{2+}$-efflux from pancreatic fragments is not inhibited after 5 min preincubation, but only after prolonged preincubation in this medium. So obviously tetraethylammonium has some effect, perhaps intracellularly, which blocks the release of calcium.

Another difference between the tetraethylammonium low sodium medium and the sucrose low sodium medium is that replacing the former

medium by normal Krebs-Ringer bicarbonate medium results in an increased enzyme release and a slight increase in $^{45}Ca^{2+}$-efflux, while replacing the latter medium by normal Krebs-Ringer bicarbonate medium results in an increased amylase release,but not in a detectable increase in $^{45}Ca^{2+}$-efflux. It is not yet clear how this disagreement can be explained.

Fig.6.9 represents a simplified scheme for the stimulus-secretion coupling process in the pancreas. Based on our results it can be concluded that incubation in a low sodium or an ouabain containing medium blocks step 2. Incubation in the tetraethylammonium low sodium medium or prolonged incubation in the sucrose low sodium medium has an additional inhibitory effect on step 1 of the stimulus-secretion coupling process.

It is not yet clear how sodium is involved in the coupling between intracellular calcium release and exocytosis. Possibly, carbachol-induced depolarization of the membrane potential, which is inhibited at low extracellular sodium concentration (Matthews and Petersen, 1973; Nishiyama and Petersen, 1975) and perhaps also by ouabain, is necessary for step 2 of stimulus-secretion coupling. This hypothesis agrees with the observation that incubation in a high potassium medium, which causes depolarization of the membrane potential, does not result in an increased amylase release (Poulsen and Williams, 1977b). This would mean that for stimulation of the enzyme secretion both intracellular calcium release and depolarization of the membrane potential are necessary.

Alternatively, it is possible that not depolarization as such, but sodium influx is required for coupling of intracellular calcium release and exocytosis. Case et al. (1978) have indeed measured a caerulein- and acetylcholine-induced dose-dependent increase in $^{22}Na^{+}$ uptake in rat pancreatic fragments. We may assume that in our experiments with a low sodium medium there is no sodium gradient across the plasma membrane. Then the increase in membrane permeability for sodium by carbachol will not result in a sodium influx. This may explain the inhibition of the stimulated enzyme secretion in the low sodium medium. However, when ouabain is used without preincubation,

there is still a sodium gradient when carbachol is added, but this will gradually disappear through the inhibition of the Na^+-K^+ATPase This may explain why in the presence of ouabain there is only a small inhibition of the stimulated enzyme secretion.

In order to investigate this tentative conclusion, we have incubated pancreatic fragments in a normal Krebs-Ringer bicarbonate medium in the presence of gramicidin or amphotericin B, which are both sodium ionophores. We find no change in the basal enzyme secretion (Renckens et al., unpublished results). Assuming that these substances will also induce a sodium influx in pancreatic acinar cells, this would indicate that a sodium influx by itself does not stimulate enzyme secretion. When carbachol is added after 30 min incubation in a medium containing one of these sodium ionophores, there is a normal stimulation of enzyme secretion. This suggests that a sodium gradient across the plasma membrane is not required for stimulation of enzyme secretion. Further investigations are necessary to provide more information about the mechanism of the inhibition of the carbachol-induced enzyme secretion observed in low sodium medium and in medium containing ouabain.

Summarizing it can be concluded that sodium ions are indeed involved in pancreatic enzyme secretion. Disturbance of the sodium metabolism of the acinar cell seems to affect the coupling between intracellular calcium release and exocytosis. However, since we do not yet know how calcium ions stimulate exocytosis, it is very difficult to indicate more explicitely how sodium ions are involved in this process.

CHAPTER VII

GENERAL DISCUSSION AND SUMMARY

The experiments described in the previous chapters have been executed in order to obtain more information about the enzyme secretion process in the exocrine pancreas. Pancreatic enzyme secretion can be stimulated by the neurotransmitter acetylcholine and by several hormones, a.o. pancreozymin and secretin. The interaction between the stimulant and its receptor on the basal plasma membrane triggers an intracellular process, eventually resulting in enzyme secretion by exocytosis. In this study we have focussed our attention on the interaction between the hormone pancreozymin and its receptor and on the role of several substances as second messenger in stimulus-secretion coupling.

It was already known that the C-terminal tetrapeptide of pancreozymin is the part of the hormone minimally required for binding to the receptor and that the C-terminal octapeptide has the same biological effects as pancreozymin itself. The tryptophan residue in position 4 from the C-terminal end is essential for the pancreatic activity of both tetragastrin (Van Nispen, 1974) and pancreozymin-C-octapeptide (Morley et al., 1965; Morley, 1968; Yabe et al., 1977). We have studied which property of tryptophan is important for binding to the receptor: charge-transfer capacity or hydrogen bonding.

Tetrafluorination of tryptophan reduces the charge-transfer capacity of tryptophan. When this tryptophan derivative is incorporated in pancreozymin-C-octapeptide instead of native tryptophan, the resulting peptide has only a small stimulatory effect on pancreatic adenylate cyclase activity, enzyme secretion by pancreatic fragments and in vivo fluid and enzyme secretion. This indicates that the charge-transfer capacity of tryptophan is necessary for complete activation of the receptor.

We have also tested the activity of pancreozymin-C-octapeptide analogues in which the capacity to form a hydrogen bond is abolished.

The biological activity of these peptides is also reduced compared to the activity of the native pancreozymin-C-octapeptide. However, when the hydrogen bond formation is abolished by replacing the nitrogen atom in the indolyl ring of tryptophan by either an oxygen or a sulfur atom, this causes also a reduction of the charge-transfer capacity. When the hydrogen bond formation is abolished by replacing the nitrogen atom by a $N(CH_3)$ group this increases the steric hindrance between the peptide and its receptor. So it is difficult to say whether the decrease in activity is caused by the absence of the capacity to form a hydrogen bond or by the side effects.

We have also observed that all four parameters used to determine the biological activity of the analogues of pancreozymin-C-octapeptide, viz adenylate cyclase activity, amylase secretion by fragments and in vivo fluid and enzyme secretion, are inhibited in parallel fashion. This means that it is very likely that occupation of a single receptor leads to the various biological effects. A similar parallel inhibition is also found by Robberecht et al. (1978), who have used several analogues of caerulein. Long and Gardner (1977), on the other hand, have observed quantitatively different effects of certain pancreozymin-C-octapeptide analogues on $^{45}Ca^{2+}$ efflux and adenylate cyclase activity, leading them to the conclusion that multiple types of receptors are involved in mediating several effects of pancreozymin.

Our experiments also show, as is generally found, that higher peptide concentrations are needed to stimulate the adenylate cyclase activity than to stimulate the amylase release from pancreatic fragments. We like to ascribe this to the presence of spare receptors: occupation of a part of the receptors leads to maximal stimulation of the amylase secretion, while for maximal stimulation of the adenylate cyclase activity a more complete occupation of the receptors is necessary. We prefer this explanation to the assumption of the presence of high- and low-affinity receptors (Robberecht et al., 1978a).

Since the hormone-receptor interaction takes place at the basal plasma membrane and the result of this interaction is exocytosis at

the apical side of the cell, it is clear that one or more second messengers are necessary to transfer the signal of the hormone-receptor interaction from one side to the other side of the cell. It is technically difficult to measure directly compounds present in very low and only transiently elevated concentrations in pancreatic acinar cells. Hence, we have attempted to use liposomes to study the role of possible second messengers. It has been shown that it is possible to introduce various substances, like drugs and enzymes, into a cell by means of liposomes (Tyrell et al., 1976; Pagano and Weinstein, 1978). Theoharides and Douglas (1978) have incubated mast cells together with liposomes containing calcium, which is supposed to be a second messenger in mast cell stimulus-secretion coupling. This incubation resulted in an increased histamine release. Gutman et al. (1979) have obtained similar result by incubating the adrenal gland in the presence of liposomes containing calcium or sodium. Unfortunately, we have not succeeded in transferring the liposome content into the pancreatic acinar cell, either because liposomes of unsuitable composition were used or because acinar cells are not suitable for such experiments. This means that all information about possible second messengers in stimulus-secretion coupling has had to be derived from indirect experiments.

At the beginning of our study there was already strong evidence for a role of calcium ions as second messenger in pancreatic enzyme secretion: stimulation of enzyme secretion causes a rise in cytoplasmic calcium concentration (Case and Clausen, 1973; Williams and Lee, 1974; Schreurs et al., 1976a) and increasing the cytoplasmic calcium concentration by means of the ionophore A23187 results in an increased enzyme release (Eimerl et al., 1974; Schreurs et al., 1976a; Poulsen and Williams, 1977).

In chapter V we have studied calcium metabolism in the acinar cell in order to obtain more information about the role of calcium ions in pancreatic stimulus-secretion coupling. We have observed that both the total and the exchangeable calcium contents rise with increasing extracellular calcium concentration. However, at all calcium concentrations tested only a minor part (< 20%) of the cellular

calcium ions exchange with $^{45}Ca^{2+}$. The unexchangeable calcium, the amount of which does not depend on the extracellular calcium concentration, is probably sequestered in the zymogen granules. Addition of carbachol to cells preloaded with $^{45}Ca^{2+}$, initially causes a $^{45}Ca^{2+}$-efflux, followed by a reuptake of $^{45}Ca^{2+}$ depending on the extracellular calcium concentration. This observation is explained in the following way: Carbachol releases calcium from an intracellular pool resulting in an increase of the cytoplasmic calcium concentration and consequently a calcium efflux. The subsequent uptake of calcium is necessary to restore calcium pools which are depleted during the stimulatory activity of the acinar cells.

Our finding that the later calcium uptake depends on the extracellular calcium concentration can be explained by the fact that stimulation of enzyme secretion also depends on the extracellular calcium concentration. When carbachol is added to a cell suspension simultaneously with $^{45}Ca^{2+}$, the stimulant causes an increased uptake of $^{45}Ca^{2+}$. This implies that carbachol causes not only a release of calcium from an intracellular pool, but also an increase in membrane permeability for calcium. The calcium release is supposed to be important for the initiation of the enzyme secretion, which is confirmed by the observation that enzyme secretion can be stimulated in a calcium-free medium. The later calcium uptake is necessary for continuation of the enzyme secretion.

Stolze and Schulz (1980) have made the interesting observation that replacement of carbachol, added to rat acinar cells preloaded with $^{45}Ca^{2+}$, by atropine causes additional uptake of $^{45}Ca^{2+}$; subsequent addition of pancreozymin again causes a $^{45}Ca^{2+}$-efflux. When carbachol is not removed before addition of pancreozymin, this hormone causes no $^{45}Ca^{2+}$-efflux. These data suggest that the calcium pool, from which calcium is released under the influence of carbachol, is refilled only when carbachol is removed from the receptor. The uptake of calcium, which occurs in the presence of carbachol, is probably necessary to replenish other calcium pools in the acinar cell.

The pool from which calcium is released upon stimulation and the nature of the coupling between hormone-receptor interaction and

this pool are not yet known. The important calcium pool may represent calcium bound to the inside of the plasma membrane or else calcium sequestered in cell organelles like mitochondria or endoplasmic reticulum. If calcium bound to the plasma membrane would represent this pool, phosphatidylinositol might play a role in the release of this calcium upon stimulation. Already in 1955 Hokin and Hokin have observed that stimulation of pancreatic enzyme secretion causes an increase in the turnover of phosphatidylinositol. Later it became clear that the increased turnover of phosphatidylinositol is initiated by breakdown of this phospholipid and that in all processes in which calcium is a second messenger this increased turnover is observed (Michell, 1975, 1979). This suggests that there is a coupling between the receptor of the stimulant and an enzyme which catalyzes the breakdown of phosphatidylinositol. Recently, Calderon et al. (1980) have observed that in rat pancreas the secretagogue-induced increase in phosphatidylinositol turnover is one of the early events in stimulus-secretion coupling. How the breakdown of phosphatidylinositol is involved in increasing the cytoplasmic calcium concentration is not yet clear. A simple, rather speculative possibility is that calcium bound to phosphatidylinositol in the plasma membrane is released when this phospholipid is metabolized, thus causing the increase in cytoplasmic calcium concentration. On the other hand, it is also possible that the increased turnover of phosphatidylinositol is involved in the continuation of the enzyme secretion. The turnover of phosphatidylinositol is considered to increase the membrane permeability for calcium, resulting in the calcium influx required for replenishing the calcium pools.

Pancreatic enzyme secretion can be stimulated by pancreozymin. This hormone stimulates the adenylate cyclase activity (Svoboda et al., 1976, 1978; Kempen et al., 1977a; De Pont et al., 1979), but causes no increase (Benz et al., 1972; Robberecht et al., 1974; Albano et al., 1976) or only a small increase in cAMP level (Deschodt-Lanckman et al., 1975; Kempen et al., 1977a). In chapter IV we have studied this discrepancy. One of the causes of this discrepancy may be an incomplete inhibition of the phosphodiesterase activity

during incubation. We have observed that in the presence of 10 mM 1-methyl-3-isobutylxanthine pancreozymin has a maximal effect on the cAMP content. The investigators who do not find a pancreozymin-induced rise in cAMP content have all used theophylline as phosphodiesterase inhibitor, which is less effective than 1-methyl-3-isobutylxanthine (Kempen et al., 1977a). Maximal stimulation of the cAMP content occurs with 10^{-6}M pancreozymin, while maximal stimulation of enzyme secretion occurs at about 10^{-8}M (see chapter II). It is generally observed that for maximal stimulation of adenylate cyclase a rather high pancreozymin concentration is necessary. This phenomenon is probably due to the presence of spare receptors.

Pancreozymin stimulates not only the adenylate cyclase activity, but has also an effect on the calcium metabolism of the acinar cells. The former effect might be antagonized by the latter one, which may cause the discrepancy between the effect of pancreozymin on adenylate cyclase activity and cAMP content. So we have checked whether influencing the calcium metatolism of the cells by varying the extracellular calcium concentration has an effect on the pancreozymin-induced cAMP content. We have found that when the extracellular calcium concentration is decreased to 1.5 mM or less, 10^{-6}M pancreozymin causes a nearly 8-fold increase in cAMP content, provided that the phosphodiesterase activity is maximally inhibited. At an extracellular calcium concentration of 2.5 mM this increase is less than 4-fold. Obviously, the mere lowering of the extracellular calcium concentration from 2.5 to 1.5 mM opposes the pancreozymin-induced rise in cytoplasmic calcium concentration, so that the stimulation of adenylate cyclase is not antagonized anymore. Because there are no indications that adenylate cyclase is directly inhibited by calcium at a concentration as may be expected in the cytoplasm, the effect is probably mediated by another component. Under normal conditions the rise in cytoplasmic calcium concentration is followed by a rise in cGMP content. Cyclic GMP may inhibit adenylate cyclase, but we have not been able to demonstrate such an effect. Hence, this possibility is not very likely.

In recent years, the presence of calmodulin, a Ca^{2+}-dependent

regulatory protein, has been demonstrated in many tissues, including the pancreas (VanderMeers et al., 1977). Calmodulin is a protein, which is activated by calcium and which in the activated form stimulates various enzymes. It can activate phosphodiesterase activity (Cheung, 1970), but this plays probably no role in our experiments because of the presence of 10 mM 1-methyl-3-isobutylxanthine. In some tissues calmodulin activates adenylate cyclase (Cheung et al., 1975), but this cannot explain our results either, for then there would be less activation of adenylate cyclase by calmodulin at a lower cytoplasmic calcium concentration and hence less cAMP, instead of more as we have found.

In addition to a role of calcium, there are several indications that sodium may also play a second messenger role in pancreatic stimulus-secretion coupling. In chapter VI we have studied the role of sodium in pancreatic enzyme secretion. When isolated acinar cells are preincubated for 60 min in a medium containing 25 mM Na^+, the carbachol-induced enzyme secretion is nearly completely abolished. Preincubation for 60 min in the presence of ouabain also has an inhibitory effect on stimulation of enzyme secretion, but much less than the low sodium medium. When pancreatic fragments are preincubated for 5 min in the above media and then stimulated with carbachol, the inhibitory effects are similar. This suggests that reduced ion fluxes across the membrane rather than changed ion contents of the cells are responsible for the inhibition.

Generally, we have used tetraethylammonium chloride to replace sodium chloride in the low sodium medium. However, because tetraethylammonium appears to penetrate the cell and thus may cause some additional effects, we have also used sucrose to replace sodium chloride. The effect on amylase secretion by pancreatic fragments is the same. However, the $^{45}Ca^{2+}$-efflux pattern in low sodium medium depends on the substance used to replace sodium. After 5 min incubation in the tetraethylammonium low sodium medium carbachol causes no $^{45}Ca^{2+}$-efflux, while in the sucrose low sodium medium the carbachol-induced $^{45}Ca^{2+}$-efflux disappears only after prolonged incubation in this medium. This means that under these conditions carbachol is not able

to release calcium from the intracellular pool. The observation that the stimulation of the enzyme secretion by A23187 in the presence of calcium is also inhibited in the low sodium medium, indicates that in this medium a step between increased cytoplasmic calcium concentration and exocytosis is also blocked. Incubation in the presence of ouabain has no effect on the carbachol-induced $^{45}Ca^{2+}$-efflux, which implies that in this medium the inhibition takes place after the release of calcium from the intracellular pool.

In our opinion it is now clear that, in addition to calcium, sodium plays a role in pancreatic stimulus-secretion coupling, but it is still uncertain whether a sodium influx or a depolarization of the plasma membrane is important for this coupling and what is the actual stimulant for exocytosis. It is difficult to study the exocytosis process by incubating isolated zymogen granules together with plasma membranes, for the zymogen granules are only stable under rather a-physiological conditions (in a sucrose medium without ions at pH 5.5; Rutten, 1974). We have unsuccessfully tried to isolate zymogen granules, which are stable in a physiological environment. If the microtubular system would be involved in pancreatic enzyme secretion, experiments in which isolated granules and plasma membranes are incubated together will not succeed. Vinblastine, which interacts with microtubules, and cytochalasin B, which affects microfilaments, both inhibit, although at rather high concentrations, the stimulated enzyme secretion in the pancreas (Bauduin et al., 1975; Williams and Lee, 1976; Stock et al., 1978). This would argue in favor of a role of the microtubular and/or microfilamentous system in stimulus-secretion coupling. However, the step at which the microtubular system would interfere is not yet known. Recently, Rochette-Egly et al. (1980) have observed that vinblastine and cytochalasin B, in the concentration in which they inhibit the stimulated enzyme secretion, also prevent the secretagogue-induced rise in cGMP content of the cells. This does not necessarily mean that cGMP mediates the effect of the microtubular system on the enzyme secretion. In the light of the rather strong evidence supplied by Gunther and Jamieson (1979) and Gardner and Rottman (1980) that cGMP plays no important

role in stimulus-secretion coupling, the observation of Rochette-Egly et al. may be regarded as a side-effect.

In recent years several investigators have studied the protein carboxyl-methylation system, present in mammalian cells, and its role in exocytosis. Gagnon and Heisler (1979) have designed a working model based on the known properties of this system. The hormone-receptor interaction causes an increase in the activity of protein carboxyl-methylase, the enzyme which catalyzes the transfer of a methyl-group from S-adenosyl-methionine to a protein. Membranes of zymogen granules appear to contain proteins, which are good substrates for protein carboxyl-methylase. Methylation of the carboxyl-group neutralizes the negative charge and would thus decrease the electrostatic repulsion between the granule and the negatively charged plasma membrane, which would promote exocytosis. However, also in this model a second messenger is necessary to link the hormone-receptor interaction at the plasma membrane to protein methylation at the granule membrane.

Ishizaka et al. (1980) have observed that in rat mast cells methylation of phospholipids in the plasma membrane is one of the early steps in stimulus-secretion coupling. After binding of the stimulant to its receptor phosphatidylethanolamine is methylated to phosphatidylcholine, which process is accompanied by translocation of the lipid in the membrane (Hirata and Axelrod, 1978). This methylation leads in the mast cells to opening of calcium channels and consequently a calcium influx, which eventually results in a stimulated histamine release. Possibly, methylation of phospholipids in the plasma membrane also plays a role in pancreatic enzyme secretion by regulating the membrane permeability for calcium or by influencing the binding of calcium to the plasma membrane.

De exocrine pancreas secreteert een sap dat, naast verschillende electrolyten, enzymen bevat die bij de spijsvertering betrokken zijn. In het hier beschreven onderzoek is het enzymsecretie proces in de pancreas nader bestudeerd. De enzymsecretie kan gestimuleerd worden door de neurotransmitter acetylcholine en door verschillende hormonen, o.a. secretine en pancreozymine. Deze stimulantia kunnen de cel niet binnendringen en geven hun prikkel af via een interactie met hun receptor op de plasmamembraan. Dit brengt een intracellulair proces op gang dat uiteindelijk resulteert in enzymsecretie via exocytose. In deze studie is de aandacht vooral gericht op de interactie tussen pancreozymine en zijn receptor en op de rol van verschillende stoffen als "second messenger" in de stimulus-secretie koppeling.

In hoofdstuk 2 is de binding tussen pancreozymine en zijn receptor bestudeerd. Het was al bekend dat het C-terminale octapeptide en daarin met name het tryptofaan-residue belangrijk is voor deze binding. Met behulp van analoga van het pancreozymine-octapeptide, waarin derivaten van tryptofaan zijn ingebouwd, is onderzocht welke eigenschap van tryptofaan belangrijk is voor de binding met de receptor. Het blijkt dat vooral het vermogen van de indol-ring van tryptofaan om lading over te kunnen dragen essentieel is voor de binding. Wanneer tryptofaan-derivaten, die geen waterstofbrug kunnen vormen, worden ingebouwd, dan is de biologische activiteit van het resulterende pancreozymine-octapeptide ook sterk verlaagd. Hieruit kan echter geen harde conclusie getrokken worden, omdat de modificatie, die de waterstofbrug vorming verhindert, tegelijkertijd ofwel een verlaging van het vermogen om lading over te dragen of een verhoging van de sterische hinder veroorzaakt.

Omdat het ons niet is gelukt d.m.v. liposomen stoffen in de acineuze cel te brengen, moest alle informatie over eventuele "second messengers" uit indirecte experimenten gehaald worden. Het feit dat pancreozymine het adenylaat cyclase stimuleert zou kunnen duiden op een rol voor cAMP als "second messenger". Maar een, zelfs geringe,

verhoging van het cAMP-gehalte van de pancreas onder invloed van pancreozymine was slechts door enkele onderzoekers gevonden. In hoofdstuk 4 hebben we deze tegenstrijdigheid onderzocht. We hebben gevonden dat een optimale remming van de fosfodiesterase activiteit noodzakelijk is om een maximaal effect van pancreozymine op het cAMP-gehalte te kunnen meten. Bovendien is gebleken dat de stijging van de cytoplasmatische calcium concentratie o.i.v. pancreozymine de stimulering van adenylaat cyclase tegenwerkt. Deze waarnemingen kunnen de tegenstrijdigheid tussen het effect van pancreozymine op de adenylaat cyclase activiteit en het cAMP-gehalte verklaren, maar geven geen uitsluitsel over een rol voor cAMP als "second messenger".

Bij de start van dit onderzoek was al bekend dat bij stimulering van de enzymsecretie een stijging van de cytoplasmatische calcium concentratie optreedt, waaruit geconcludeerd werd dat Ca^{2+}-ionen een "second messenger" rol vervullen in de stimulus-secretie koppeling in de pancreas. Uit onze experimenten is gebleken dat carbachol, een analogon van acetylcholine, het calciummetabolisme op twee manieren beinvloedt. 1) Carbachol maakt calcium vrij uit een intracellulaire pool, hetgeen leidt tot een stijging van de cytoplasmatische calcium concentratie. 2) Carbachol verhoogt de membraanpermeabiliteit voor calcium, hetgeen leidt tot een calcium-influx. Het eerste proces is belangrijk voor de initiatie van de enzymsecretie. Dit wordt bevestigd door het feit dat ook in calcium-vrij medium de enzymsecretie gestimuleerd kan worden. Het tweede proces speelt een rol bij de continuering van de enzymsecretie. Het calcium dat de cel instroomt is nodig om calcium-pools, die uitgeput raken tijdens het enzymsecretie proces, weer op te vullen.

Omdat er aanwijzingen waren, o.a. op grond van electrofysiologische experimenten, dat ook Na^{+}-ionen betrokken zijn bij de enzymsecretie in de pancreas, hebben we in hoofdstuk 6 de rol van natrium in de stimulus-secretie koppeling bestudeerd. Geisoleerde acineuze cellen en pancreas-fragmenten zijn geincubeerd in medium met een verlaagde natrium concentratie en in medium met ouabaine. Onder deze omstandigheden is het effect van carbachol op de enzymsecretie en het calciummetabolisme van de acineuze cel gemeten. Uit deze experi-

menten blijkt dat door verlaging van de extracellulaire natrium concentratie en door toevoeging van ouabaine aan het medium de koppeling tussen de gestegen cytoplasmatische calcium concentratie en de exocytose geblokkeerd is. Incubatie in medium met lage natrium concentratie kan, afhankelijk van de stof die gebruikt is om natrium te vervangen en van de preincubatietijd, ook de koppeling tussen hormoon-receptor interactie en stijging van de cytoplasmatische calcium concentratie blokkeren.

Op grond van onze gegevens hebben we geconcludeerd dat zowel Ca^{2+}- als Na^{+}-ionen betrokken zijn bij de enzymsecretie in de pancreas. Het is echter nog niet duidelijk welke stappen achtereenvolgens optreden in de stimulussecretie koppeling. In hoofdstuk 7 hebben we, aan de hand van recente litteratuur, hiervoor enkele suggesties gedaan. Verder onderzoek is nodig om deze suggesties te toetsen.

Adelson, J.W. and Rothman, S.S. (1975) Chymodenin, a duodenal peptide: specific stimulation of chymotrypsinogen secretion. Amer.J. Physiol. 229, 1680-1686.

Albano, J., Bhoola, K.D. and Harvey, R.F. (1976) Intracellular messenger role of cyclic GMP in exocrine pancreas. Nature 262, 404-406.

Albinus, M., Blair, E.L., Case, R.M., Coy, D.H., Gomez-Pan, A., Hirst, B.H., Reed, J.D., Schally, A.V., Shaw, B., Smith, P.A. and Smy, J.R. (1977) Comparison of the effect of somatostatin on gastrointestinal function in the conscious and anaesthetized cat and on the isolated cat pancreas. J.Physiol. 269, 77-91.

Alving, C.R. and Steck, E.A. (1979) The use of liposome-encapsulated drugs in leishmaniasis. TIBS 4, N175-N177.

Amsterdam, A. and Jamieson, J.D. (1974) Studies on dispersed pancreatic exocrine cells. I. Dissociation technique and morphologic characteristics of separated cells. J.Cell.Biol. 63, 1037-1056.

Anastasi, A., Erspamer, V. and Endeau, R. (1967) Isolation and structure of caerulein, an active decapeptide from the skin of Hyla Caerulea. Experientia 23, 699-700.

Anastasi, A., Bernardi, L., Bertaccini, G., Bosisio, G., De Castiglione, R., Erspamer, V., Goffredo, O. and Impicciatore, G. (1968) Synthetic peptides related to caerulein. Experientia 24, 771-773.

Argent, B.E., Case, R.M. and Scratcherd, T. (1973) Amylase secretion by the perfused cat pancreas in relation to the secretion of calcium and other electrolytes and as influenced by the external ionic environment. J.Physiol. 230, 575-593.

Arimura, A., Sato, H., Dupont, A., Nishi, N. and Schally, A.V. (1975) Somatostatin: abundance of immunoreactive hormone in rat stomach and pancreas. Science 189, 1007-1009.

Baker, P.F. (1977) Calcium and the control of neuro-secretion. Sci.Prog.Oxf. 64, 95-115.

Ball,E.G. and Johnston, C.G. (1930) Variations in inorganic constituents of the pancreatic juice during constant drainage of the pancreatic ducts. J.Biol.Chem. 86, 643-653.

Bauduin, H., Stock, C., Vincent, D. and Grenier, J.F. (1975) Microfilamentous system and secretion of enzyme in the exocrine pancreas. Effect of cytochalasin B. J.Cell.Biol. 66, 165-181.

Benz, L., Eckstein, B., Matthews, E.K. and Williams, J.A. (1972) Control of pancreatic amylase release in vitro: effects of ions, cyclic AMP and colchicine. Br.J.Pharmacol. 46, 66-77.

Berg, N.B. and Young, R.W. (1971) Sulfate metabolism in pancreatic acinar cells. J.Cell Biol. 50, 469-483.

Bergeron, J.J.M., Sikstrom, R., Hand, A.R. and Posner, B.I. (1979) Binding and uptake of ^{125}I-insulin into rat liver hepatocytes and endothelium. An in vivo radioautographic study. J.Cell Biol. 80, 427-443.

Bernfeld, P. (1955) In: Methods in Enzymology, Colowick, S.P. and Kaplan, N.O. (eds) Vol.I, pp 149-158, Academic Press, New York.
Birdsall, N.J.M., Burgen, A.S.V., Hulme, E.C. and Wells, J.W. (1979) The effects of ions on the binding of agonists and antagonists to muscarinic receptors. Br.J.Pharmac. 67, 371-377.
Blaustein, M. (1974) The interrelationship between sodium and calcium fluxes across cell membranes. Rev.Physiol.Biochem.Pharmacol. 70, 33-82.
Bloom, W. and Fawcett, D.W. (1975) In: A textbook of histology. W.B.Saunders Co, Philadelphia, pp 726-742.
Bodanszky, M., Klausner, Y.S. and Said, S.I. (1973) Biological activities of synthetic peptides corresponding to fragments of and to the entire sequence of the vasoactive intestinal peptide. Proc.Nat.Acad.Sci. USA 70, 382-384.
Bodanszky, M., Natarajan, S., Hahne, W. and Gardner, J.D. (1977) Cholecystokinin (pancreozymin) 3. Synthesis and properties of an analogue of the C-terminal heptapeptide with serine sulfate replacing tyrosine sulfate. J.Med.Chem. 20, 1047-1050.
Bodanszky, M., Martinez, J., Priestley, G.P., Gardner, J.D. and Mutt, V. (1978) J.Med.Chem. 21, 1030-1035.
Boden, G., Sivitz, M.C. and Owen, O.E. (1975) Somatostatin suppresses secretin and pancreatic exocrine secretion. Science 190, 163-165.
Bonting, S.L. (1970) Sodium-potassium activated adenosinetriphosphatase and cation transport. In: Membranes and ion transport. Bittar, E.E. ed. Wiley Interscience London, pp 257-363.
Bonting, S.L., De Pont, J.J.H.H.M., Kempen, H.J.M., Case, R.M., Smith, P.A. and Scratcherd, T. (1977) The effects of alloxanate, nicotinic acid and imidazole on secretory processes and the activities of adenylate cyclase and 3',5'-AMP phosphodiesterase in cat pancreas. Br.J.Pharmac. 61, 243-250.
Bonting, S.L., De Pont, J.J.H.H.M. and Jansen, J.W.C.M. (1980) Role of sodium ions in pancreatic fluid secretion. J.Physiol.
Brown, B.L., Albano, J.D.M., Ekins, R.P. and Sgherzi, A.M. (1971) A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. Biochem.J. 121, 561-563.
Bussolati, G., Capella, C.,Solcia, E., Vassalo, G. and Vezzadini, P. (1971) Ultrastructural and immunofluorescent investigations of the secretin cell in the dog intestinal mucosa. Histochemie 26, 218-227.
Calderon, P., Furnelle, J. and Christophe, J. (1980) Phosphatidylinositol turnover and calcium movement in the rat pancreas. Amer.J.Physiol. 238, G247-G254.
Campbell, P.N. and Blobel, G. (1976) The role of organelles in the chemical modification of the primary translation products of secretory proteins. FEBS Letters 72, 215-226.
Case, R.M., Harper, A.A. and Scratcherd, T. (1968) Water and electrolyte secretion by the perfused pancreas of the cat. J.Physiol. 196, 133-149.
Case, R.M., Scratcherd, T. and Wynne, R.D.A. (1970) The origin and secretion of pancreatic juice bicarbonate. J.Physiol. 210, 1-15.

Case, R.M., Johnson, M., Scratcherd, T. and Sherratt, H.S.A. (1972) Cyclic adenosine 3',5'-monophosphate concentration in the pancreas following stimulation by secretin, cholecystokinin pancreozymin and acetylcholine. J.Physiol. 223, 669-684.
Case, R.M. and Scratcherd, T. (1972) The action of dibutyryl cyclic adenosine 3',5'-monophosphate and methylxanthines on pancreatic exocrine secretion. J.Physiol. 223, 649-667.
Case, R.M. (1973) Cellular mechanisms controlling pancreatic exocrine secretion. Acta Hepato-Gastroenterologica 20, 435-443.
Case, R.M. and Clausen, T. (1973) The relationship between calcium exchange and enzyme secretion in the isolated rat pancreas. J.Physiol. 235, 75-102.
Case, R.M. and Scratcherd, T. (1974) The secretion of alkali metal ions by the perfused cat pancreas as influenced by the composition and osmolarity of the external environment and by inhibitors of metabolism and Na^+,K^+-ATPase activity. J.Physiol. 242, 415-428.
Case, R.M. (1978) Synthesis, intracellular transport and discharge of exportable proteins in the pancreatic acinar cell and other cells. Biol.Rev. 53, 211-354.
Case, R.M., Clausen, T. and Scott-Wilson, C.J. (1978) Extracellular sodium and stimulus-secretion coupling in rat exocrine pancreas. J.Physiol. 284, 47P-48P.
Case, R.M., Hotz, J., Hutson, D., Scratcherd, T. and Wynne,R.D.A. (1979) Electrolyte secretion by the isolated cat pancreas during replacement of extracellular bicarbonate by organic anions and chloride by inorganic anions. J.Physiol. 286, 563-576.
Casteels, R. and Van Breemen, C. (1975) Active and passive Ca^{2+} fluxes across cell membranes of the guinea pig taenia coli. Pflüg.Arch.Gesamte Physiol. Menschen Tiere 359, 197-207.
Chandler, D.E. and Williams, J.A. (1977) Intracellular uptake and α-amylase and lactate dehydrogenase releasing actions of the divalent cation ionophore A23187 in dissociated pancreatic acinar cells. J.Membrane Biol. 32, 201-230.
Chandler, D.E. and Williams, J.A. (1978a) Intracellular divalent cation release in pancreatic acinar cells during stimulus-secretion coupling. I. Use of chlorotetracycline as fluorescent probe. J.Cell Biol. 76, 371-385.
Chandler, D.E. and Williams, J.A. (1978b) Intracellular divalent cation release in pancreatic acinar cells during stimulus-secretion coupling. II. Subcellular localization of the fluorescent probe chlorotetracycline. J.Cell Biol. 76, 386-399.
Cheung, W.Y. (1970) Cyclic 3',5'-nucleotide phosphodiesterase. Demonstration of an activator. Biochem.Biophys.Res.Commun. 38, 533-538.
Cheung, W.Y., Bradham, L.S., Lynch, T.J., Ling, Y.M. and Tallant, E.A. (1975) Protein activator of cyclic 3';5'-nucleotide phosphodiesterase of bovine or rat brain also activates its adenylate cyclase. Biochem.Biophys.Res.Commun. 66, 1055-1062.
Christophe, J.P., Conlon, T.P. and Gardner, J.D. (1976a) Interaction of porcine vasoactive intestinal peptide with dispersed pancreatic acinar cells from the guinea pig. J.Biol.Chem. 251,

4629-4634.
Christophe, J.P., Frandsen, E.F., Conlon, Th.P., Krishna, G. and Gardner, J.D. (1976b) Action of cholecystokinin, cholinergic agents, and A23187 on accumulation of guanosine 3',5'-monophosphate in dispersed guinea pig pancreatic acinar cells. J.Biol. Chem. 251, 4640-4645.
Clemente, F. and Meldolesi, J. (1975) Calcium and pancreatic secretion-dynamics of subcellular calcium pools in resting and stimulated acinar cells. Br.J.Pharmacol. 55, 369-379.
Cole, W.H., Allison, J.B., Murray, T.J., Boyden, A.A., Anderson, J.A. and Leathen, J.H. (1944) Amer.J.Physiol. 141, 165-171.
Coy, D.H., Coy, E.J., Hirotsu, Y., Vilchez-Martinez, J.A., Schally, A.V., Van Nispen, J.W. and Tesser, G.I. (1974) Investigation of the role of tryptophan in the luteinizing hormone-releasing hormone. Biochemistry 13, 3550-3553.
Davis, B. and Lazarus, N.R. (1976) An in vitro system for studying insulin release caused by secretory granules-plasma membrane interaction: definition of the system. Biochim.Biophys.Acta 443, 437-442.
Dean, P.M. (1975) Exocytosis modelling: an electrostatic function for calcium in stimulus-secretion coupling. J.Theoret.Biol. 54, 289-308.
De Pont, J.J.H.H.M., Luyben, D. and Bonting, S.L. (1979) Rat pancreas adenylate cyclase. VI. Role of the enzyme in secretin stimulated enzyme secretion. Biochim. Biophys. Acta 584, 33-42.
Deschodt-Lanckman, M., Robberecht, P., De Neef, P., Labrie, F. and Christophe, J. (1975) In vitro interactions of gastrointestinal hormones on cyclic adenosine 3',5'-monophosphate levels and amylase output in the rat pancreas. Gastroenterology 68, 318-325.
Deschodt-Lanckman, M., Robberecht, P., De Neef, P., Lammens, M. and Christophe, J. (1976) In vitro action of bombesin and bombesin-like peptides on amylase secretion, calcium efflux and adenylate cyclase activity in rat pancreas. Comparison with other secretagogues. J.Clin.Invest. 58, 891-898.
Dockray, G.J. (1972) The action of secretin, cholecystokinin-pancreozymin and caerulein on pancreatic secretion in the rat. J. Physiol. 225, 679-692.
Dockray, G.J., Gregory, R.A., Hutchinson, J.B., Harris, J.I. and Runswick, M.J. (1978) Isolation, structure and biological activity of two cholecystokinin octapeptides from sheep brain. Nature 274, 711-713.
Domscke, S., Domscke, W., Rösch, W., Konturek, S.J., Sprügel, W., Mitznegg, P., Wünsch, E. and Demling, L. (1977) Vasoactive intestinal peptide: a secretin-like partial agonist for pancreatic secretion in man. Gastroenterology 73, 478-480.
Douglas, W.W. (1976) The role of calcium in stimulus-secretion coupling. In: Stimulus-secretion coupling in the gastrointestinal tract. R.M.Case and H.Goebell, Eds., MTP Press, Lancaster, pp 17-31.
Dreiling, D.A. and Janowitz, H.D. (1956) The secretion of electrolytes by the human pancreas. Gastroenterology 30, 382-390.
Dunham, Ph., Babiarz, P., Israel, A., Zerial, A. and Weissmann, G.

(1977) Membrane fusion: studies with a calcium-sensitive dye, arsenazo III, in liposomes. Proc at.Acad.Sci. USA 74, 1580-1584.
Eagle, H. (1959) Amino acid metabolism in mammalian cell cultures. Science 130, 432-437.
Eimerl, S., Savion, N., Heichal, O. and Selinger, Z. (1974) Induction of enzyme secretion in rat pancreatic slices using the ionophore A23187 and calcium. J.Biol.Chem. 249, 3991-3993.
Ekholm, R., Zelander, T. and Edlund, Y. (1962a) The ultrastructural organization of the rat exocrine pancreas. I. Acinar cells. J.Ultrastruct.Res. 7, 61-72.
Ekholm, R., Zelander, T. and Edlund, Y. (1962b) The ultrastructural organization of the rat exocrine pancreas. II. Centro-acinar cells, intercalary and intralobular ducts. J.Ultrastruct.Res. 7, 73-83.
Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J.Biol.Chem. 226, 497-509.
Fölsch, U.R., Fisher, H., Hottenbacher, M. and Creutzfeldt, W. (1977) Cyclic-AMP in pancreatic duct fragments of the rat: the effect of gastrointestinal hormones. Irish J.Med.Sci., suppl.6.
Fölsch, U.R., Creutzfeldt, W. (1977) Pancreatic duct cells in rats: secretory studies in response to secretin, cholecystokinin-pancreozymin, and gastrin in vivo. Gastroenterology 73, 1053-1059.
Fölsch, U.R., Lankisch, P.G. and Creutzfeldt, W. (1978) Effect of somatostatin on basal and stimulated pancreatic secretion in the rat. Digestion 17, 194-203.
Gagnon, C. and Heisler, S. (1979) Protein carboxyl-methylation: role in exocytosis and chemotaxis. Life Sci. 25, 993-1000.
Gardner, J.D., Conlon, Th.P., Klaeveman, H.L., Adams, T.D. and Ondetti, M.A. (1975) Action of cholecystokinin and cholinergic agents on calcium transport in isolated pancreatic acinar cells. J.Clin.Invest. 56, 366-375.
Gardner, J.D. and Hahne, W.F. (1977) Calcium transport in dispersed acinar cells from rat pancreas. Biochim.Biophys.Acta 471, 466-476.
Gardner, J.D. and Jackson, M.J. (1977) Regulation of amylase release from dispersed pancreatic acinar cells. J.Physiol. 270, 439-454.
Gardner, J.D. and Rottman, A.J. (1980) Evidence against cyclic GMP as a mediator of the actions of secretagogues on amylase release from guinea pig pancreas. Biochim.Biophys.Acta 627, 230-243.
Gaskin, F., Cantor, C.R. and Shelanski, M.L. (1975) Biochemical studies on the in vitro assembly and disassembly of microtubules. Ann.New York Acad.of Sci. 253, 133-146.
Geuze, J.J. and Kramer, M.F. (1974) Function of coated membranes and multivesicular bodies during membrane regulation in stimulated exocrine pancreas cells. Cell and Tiss.Res. 156, 1-20.
Greenwell, J.R. (1975) The effects of cholecystokinin-pancreozymin, acetylcholine and secretin on the membrane potentials of mouse pancreatic cells in vitro. Pflügers Arch. 353, 159-170.

Grossman, M.I. and Ivy, A.C. (1946) Effect of alloxan upon external secretion of pancreas. Proc.Soc.Exp.Biol.Med. 63, 62-63.
Guelrud, M., Rudick, J., Janowitz, H.D. (1972) Effects of some inhibitors of sodium transport (adenosine triphosphatase inhibitors) on pancreatic secretion. Gastroenterology 62, 540-546.
Gunther, G.R. and Jamieson, J.D. (1979) Increased intracellular cyclic GMP does not correlate with protein discharge from pancreatic acinar cells. Nature 280, 318-320.
Gutman, Y., Lichtenberg, D., Cohen, J. and Boonyaviroj, P. (1979) Increased catecholamine release from adrenal medulla by liposomes loaded with sodium or calcium ions. Biochem.Pharmacol. 28, 1209-1211.
Hanssen, L.E., Hanssen, K.F. and Myren, J. (1977) Inhibition of secretin release and pancreatic bicarbonate secretion by somatostatin infusion in man. Gastroenterology 12, 391-394.
Harper, A.A. (1967) Hormonal control of pancreatic secretion. In: Handbook of Physiology, Section 6: alimentary canal, Volume II: Secretion. W.Heidel, ed. American Physiological Society, Washington. pp 969-995.
Haymovits, A. and Scheele, G.A. (1976) Cellular cyclic nucleotides and enzymes secretion in the pancreatic acinar cell. Proc.Nat. Acad.Sci. USA 73, 156-160.
Hegre, O.D., McEvoy, R.C., Bachelder, V. and Lazarow, A. (1972) Organ culture of fetal rat pancreas: quantitative analysis by linear scanning of islet and other components. In Vitro J. Tissue Cult.Ass. 7, 366-376.
Heisler, S., Fast, D. and Tenenhouse, A. (1972) Role of Ca^{2+} and cyclic AMP in protein secretion from rat exocrine pancreas. Biochim.Biophys.Acta 279, 561-572.
Heisler, S. and Grondin, G. (1973) Effect of lanthanum on ^{45}Ca flux and secretion of protein from rat exocrine pancreas. Life Sci. 13, 783-794.
Hendriks, T., Daemen, F.J.M. and Bonting, S.L. (1974) Biochemical aspects of the visual process. XXV Light-induced calcium movements in isolated frog rod outer segments. Biochim.Biophys.Acta 345, 468-473.
Hendriksen, F.W. (1968) Effect of pancreozymin on the canine pancreatic secretion of fluid and bicarbonate. Scand.J.Gastroenterol. 3, 637-640.
Hirata, F. and Axelrod, J. (1978) Enzymatic synthesis and rapid translocation of phosphatidylcholine by two methyltransferases in erythrocyte membranes. Proc.Nat.Acad.Sci.USA 75, 2348-2352.
Hoekstra, D., Tomasini, R. and Scherphof, G. (1978) Interaction of phospholipid vesicles with rat hepatocytes in primary monolayer culture. Biochim.Biophys.Acta 542, 456-469.
Hokin, L.E., Hokin, M.R. (1955) Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices and brain cortex slices. Biochim.Biophys.Acta 18, 102-110.
Hokin, L.E. (1966) Effect of calcium omission on acetylcholine stimulated amylase secretion and phospholipid synthesis in pigeon pancreas slices. Biochim.Biophys.Acta 115, 219-221.
Ishizaka, T., Hirata, F., Ishizaka, K. and Axelrod, J. (1980) Stim-

ulation of phospholipid methylation, Ca^{2+} influx, and histamine release by bridging of IgE receptors on rat mast cells. Proc. Nat.Acad.Sci. USA 77, 1903-1906.
Jamieson, J.D. and Palade, G.E. (1967) Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules. J.Cell.Biol. 34, 597-615.
Jamieson, J.D. and Palade, G.E. (1968) Intracellular transport of secretory proteins in the pancreatic exocrine cell. IV. Metabolic requirements. J.Cell.Biol. 39, 589-603.
Jamieson, J.D. and Palade, G.E. (1971) Condensing vacuole conversion and zymogen granule discharge in pancreatic exocrine cells: metabolic studies. J.Cell.Biol. 48, 503-522.
Janowitz, H.D. (1967) Pancreatic secretion of fluid and electrolytes. In: Handbook of physiology, Section 6: Alimentary canal, Volume II: Secretion. W.Heidel, ed. American Physiological Society. Washington, D.C., pp 925-933.
Jansen, J.W.C.M. (1980) Role of the paracellular pathway in pancreatic fluid secretion. Thesis, University of Nijmegen. The Netherlands.
Jensen, S.L., Fahrenkrug, J., Holst, J.J., Kühl, C., Nielsen, O.V. and Schaffalitzky de Muckadell, O.B. (1978) Secretory effects of secretin on isolated perfused porcine pancreas. Amer.J. Physiol. 235, E381-E386.
Jensen, R.T. and Gardner, J.D. (1979) Interaction of physalaemin, substance P, and eledoisin with specific membrane receptors on pancreatic acinar cells. Proc.Nat.Acad.Sci. USA 76, 5679-5683.
Kaiser, M.H. and Grossman, M.I. (1954) Pancreatic secretion in dogs with ethionine induced pancreatitis. Gastroenterology 26, 189-197.
Kanno, T. (1975) The electrogenic sodium pump in the hyperpolarizing and secretory effects of pancreozymin in the pancreatic acinar cell. J.Physiol. 245, 599-616.
Kanno, T., Saito, A. and Sato, Y. (1977) Stimulus-secretion coupling in pancreatic acinar cells: influences of external sodium and calcium on responses to cholecystokinin pancreozymin and ionophore A23187. J.Physiol. 270, 9-28.
Kanno, T. and Yamamoto, J. (1977) Differentiation between calcium-dependent effects of cholecystokinin-pancreozymin and bicarbonate-dependent effects of secretin in exocrine secretion of the rat pancreas. J.Physiol. 264, 787-799.
Kapoor, C.L. and Krishna, G. (1977) Hormone-induced cyclic guanosine monophosphate secretion from guinea pig pancreatic lobules. Science 196, 1003-1005.
Kayasseh, L., Gyr, K., Stalder, G.A., Rittmann, W.W. and Girard, J. (1978) Effect of somatostatin on exocrine pancreatic secretion stimulated by pancreozymin,secretin or by a test meal in the dog. Hormone Res. 9, 176-184.
Kempen, H.J.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1974) Rat pancreas adenylate cyclase. II. Inactivation and protection of its hormone receptor sites. Biochim.Biophys.Acta 370, 573-584.
Kempen, H.J.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1975) Rat

pancreas adenylate cyclase. III. Its role assessed by means of cholera toxin. Biochim.Biophys.Acta 392, 276-287.
Kempen, H.J.M., De Pont, J.J.H.H.M.,and Bonting, S.L. (1977a) Rat pancreas adenylate cyclase. IV. Effects of hormones and other agents on cyclic AMP level and enzyme release. Biochim.Biophys. Acta 496, 65-76.
Kempen, H.J.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1977b) Rat pancreas adenylate cyclase. V. Its presence in isolated rat pancreatic acinar cells. Biochim.Biophys. Acta 496, 521-531.
Kendall, M.G. (1948) In: Rank correlation methods. Griffin, C. and Co., London.
Kern, H.F. and Ferner, H. (1971) Die feinstruktur des exokrinen pancreasgewebe von Menschen. Z.Zellforsch. 113, 322-343.
Kolata, G.B. (1978) Polypeptide hormones: What are they doing in cells? Science 201, 895-897.
Kondo, S. and Schulz, I. (1976a) Calcium ion uptake in isolated pancreas cells induced by secretagogues. Biochim.Biophys.Acta 419, 76-92.
Kondo, S. and Schulz, I. (1976b) Ca^{2+} fluxes in isolated cells of rat pancreas. Effects of secretagogues and different Ca^{2+} concentrations. J.Membr.Biol. 29, 185-203.
Konturek, S.J., Pucher, A. and Radecki, T. (1976a) Comparison of vasoactive intestinal peptide and secretin in stimulation of pancreatic secretion. J.Physiol. 255, 497-509.
Konturek, S.J., Tasler, J., Obtulowicz, W., Coy, D.H. and Schally, A.V. (1976b) Effect of growth hormone-release inhibiting hormone on hormones stimulating exocrine pancreatic secretion. J.Clin. Invest. 58, 1-6.
Kramer, M.F. and Geuze, J.J. (1974) Redundant cell-membrane regulation in the exocrine pancreas cell after polocarpine stimulation of the secretion. In: Cytopharmacology of Secretion. B.Ceccarelli, J.Meldolesi, F.Clementi, eds. Raven Press, New York, pp 87-97.
Krishna, G., Weiss, B. and Brodie, B.B. (1968) A simple, sensitive method for the assay of adenyl cyclase. J.Pharmacol.Exp.Ther. 163, 379-385.
Kubowitz, F. and Ott, P. (1943) Isolierung und Kristallisation eines Gärungsfermentes aus Tumoren. Biochem.Z. 314, 94.
Lambert, M., Camus, J. and Christophe, J. (1975) Phosphorylation in vitro of proteins in the pancreas and parotid of rats: effects of hormonal secretagogues and cyclic nucleotides. Bioch.Pharmacol. 24, 1755-1758.
Long, B.W. and Gardner, J.D. (1977) Effects of cholecystokinin on adenylate cyclase activity in dispersed pancreatic acinar cells. Gastroenterology 73, 1008-1014.
Lopatin, R.N. and Gardner, J.D. (1978) Effects of calcium and chelating agents on the ability of various agonists to increase cyclic GMP in pancreatic acinar cell. Biochim.Biophys.Acta 543, 465-475.
Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L. (1951) Protein measurements with the folin phenol reagent. J.Biol.Chem. 193, 265-275.
Ma, T.S. and Bose, D. (1977) Sodium in smooth muscle relaxation.

J.Physiol. 232, C59-C66.
Makhlouf, G.M. (1974) The neuroendocrine design of the gut. The play of chemicals in a chemical playground. Gastroenterology 67, 159-184.
Marois, C., Morisset, J. and Dunningan, J. (1972) Presence and stimulation of adenyl cyclase in pancreatic homogenate. Rev.Can. Biol. 31, 253-257.
Matsuzaki, S. and Dumont, J.E. (1972) Effect of calcium ion on horse parathyroid gland adenyl cyclase. Biochim.Biophys.Acta 284, 227-234.
Matthews, E.K. and Petersen, O.H. (1973) Pancreatic acinar cells: ionic dependence of the membrane potential and acetylcholine induced depolarization. J.Physiol. 231, 283-295.
Matthews, E.K., Petersen, O.H. and Williams, J.A. (1973) Pancreatic acinar cells: acetylcholine-induced membrane depolarization, calcium efflux and amylase release. J.Physiol. 234, 689-701.
May, R.J., Conlon, T.P., Erspamer, V. and Gardner, J.D. (1978) Actions of peptides isolated from amphibian skin on pancreatic acinar cells. Amer.J.Physiol. 235, E112-E118.
Meyers, C.A., Coy, D.H., Huang, W.Y., Schally, A.V. and Redding, T.W. (1978) Highly active position eight analogues of somatostatin and separation of peptide diastereomers by partition chromatography. Biochemistry 17, 2326-2331.
Michell, R.H. (1975) Inositol phospholipids and cell surface receptor function. Biochim.Biophys.Acta 415, 81-147.
Michell, R.H. (1979) Inositol phospholipids in membrane function. TIBS 4, 128-131.
Milutinovic, S., Schulz, I., Rosselin, G. and Fasold, H. (1977) The interaction of pancreatic secretagogues with pancreatic plasma membranes. In: Hormone receptors in digestive tract physiology. S.Bonfils, P.Fromageot, G.Rosselin, eds., North-Holland Publishing Company, Amsterdam, pp 213-226.
Morisset, J. and Poirier, G.G. (1977) Comparative inhibitory effects of 3-quinuclidinyl benzilate (QNB) and atropine on amylase release from rat pancreas. Br.J.Pharmac. 61, 97-100.
Morley, J.S., Tracey, H.J. and Gregory, R.A. (1965) Structure-function relationships in the active C-terminal tetrapeptide sequence of gastrin. Nature 207, 1356-1359.
Morley, J.S. (1968) Structure-function relationships in gastrin-like peptides. Proc.Roy.Soc.B. 170, 97-111.
Morley, J.S. (1977) Information about peptide hormone receptors from structure-activity studies. In: First International Symposium on hormonal Receptors in Digestive Tract Physiology. S.Bonfils, P.Fromageot and G.Rosselin, eds. North-Holland Publishing Company, Amsterdam. pp 213-226.
Muller, J.E., Straus, E. and Yalow, R.S. (1977) Immunohistochemical localization in rabbit brain of a peptide resembling the COOH-terminal octapeptide of cholecystokinin. Proc.Nat.Acad.Sci. USA 74, 3035-3037.
Mutt, V. and Jorpes, J.E. (1968) Structure of porcine cholecystokinin-pancreozymin. I. Clearage with thrombin and with trypsin. Eur.J.Biochem. 6, 156-162.

Nishiyama, A. and Petersen, O.H. (1975) Pancreatic acinar cells: ionic dependence of acetylcholine-induced membrane potential and resistance change. J.Physiol. 244, 431-465.
Ondetti, M.A., Pluščes, J., Sabo, E.F., Sheenan, J.T. and Williams, M. (1970) Synthesis of cholecystokinin-pancreozymin. I. The C-terminal dodecapeptide. J.Am.Chem.Soc. 92, 195-199.
Ondetti, M.A., Rubin, B., Engel, S.L., Plusces, J. and Sheenan, J.T. (1970) Cholecystokinin-pancreozymin: recent developments. Am. J.Dig.Dis. 15, 149-156.
Pagano, R.E. and Weinstein, J.N. (1978) Interactions of liposomes with mammalian cells. Ann.Rev.Biophys.Bioeng. 7, 435-468.
Palade, G.E., Siekevitz, Ph. and Caro, L.G. (1962) Structure, chemistry and function of the pancreatic exocrine cell. In: Ciba Found. Symp. The exocrine pancreas. A.V.S.de Renck, M.P.Cameron, eds. Churchill, London. pp 23-49.
Palade, G. (1975) Intracellular aspects of the process of protein synthesis. Science 189, 347-358.
Pangborn, M.C. (1951) A simplified purification of lecithin. J.Biol. Chem. 188, 471-476.
Papahadjopoulos, D., Mayhew, E., Poste, G., Smith, S. and Vail, W.J. (1974) Incorporation of lipid vesicles by mammalian cells provides a potential method for modifying cell behaviour. Nature 252, 163-165.
Peikin, S.R., Costenbader, C.L. and Gardner, J.D. (1979) Actions of derivatives of cyclic nucleotides on dispersed acini from guinea pig pancreas. J.Biol.Chem. 254, 5321-5327.
Petersen, O.H. and Ueda, N. (1975) Pancreatic acinar cells: effects of acetylcholine, pancreozymin, gastrin and secretin on membrane potential and resistance in vivo and in vitro. J.Physiol. 247, 461-471.
Petersen, O.H. and Ueda, N. (1976) Pancreatic acinar cells: the role of calcium in stimulus-secretion coupling. J.Physiol. 254, 583-606.
Petersen, O.H. and Ueda, N. (1977) Secretion of fluid and amylase in the perfused rat pancreas. J.Physiol. 264, 819-835.
Petersen, O.H. and Philpott, H.G. (1979) dBcGMP-competitive inhibitor of CCK on electrical responses in acini. Danish Med.Bull. 26, suppl.1, p 1.
Philpott, H.G. and Petersen, O.H. (1979) Extracellular but not intracellular application of peptide hormones activates pancreatic acinar cells. Nature 281, 684-686.
Polak, J.M., Bloom, S.R., Joffe, S. and Pearse, A.G.E. (1975) Morphology and physiology of GH-RIH (somatostatin) in the gastrointestinal tract. Clin.Sci. 49, 26P.
Poste, G., Papahadjopoulos, D. and Vail, W.J. (1976) Lipid vesicles as carriers for introducing biologically active materials in cells. In: Methods in Cell Biology. D.M.Prescott, ed. Academic Press, New York, vol.14, p 33.
Poulsen, J.H. and Williams, J.A. (1977a) Effects of the calcium ionophore A23187 on pancreatic acinar cell membrane potentials and amylase release. J.Physiol. 264, 323-339.
Poulsen, J.H. and Williams, J.A. (1977b) Effect of extracellular K^+

concentration on resting potential, caerulein-induced depolarization and amylase release from mouse pancreatic acinar cells. Pflüg. Arch. 370, 173-177.
Prince, W.T., Berridge, M.J. and Rasmussen, H. (1972) The role of calcium in the action of 5-hydroxytryptamine and cyclic AMP on salivary gland. Proc.Nat.Acad.Sci. USA 69, 553-557.
Rajh, H.M., Uitzetter, J.H., Westerhuis, L.W., Van den Dries, C. and Tesser, G.I. (1979) Synthesis, resolution and charge-donor properties of six tryptophan analogues. Int.J.Peptide Protein Res. 14, 68-79.
Rajh, H.M., Mariman, E.C., Tesser, G.I. and Nivard, R.J.F. (1980) Tryptophan replacement in the C-terminal octapeptide of CCK-PZ. Int.J.Pept.Protein Res. 15, 200-210.
Rajh, H.M., Smyth, M.J., Renckens, B.A.M., Jansen, J.W.C.M., De Pont, J.J.H.H.M., Bonting, S.L., Tesser, G.I. and Nivard, R.J.F. (in the press) Role of tryptophan in the interaction of pancreozymin with its receptor. Biochim.Biophys.Acta.
Renckens, B.A.M., Schrijen, J.J., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1978) Role of calcium in exocrine pancreatic secretion. IV. Calcium movements in isolated acinar cells of rabbit pancreas. Biochim.Biophys.Acta 544, 338-350.
Renckens, B.A.M., Van Emst-De Vries, S.E., De Pont, J.J.H.H.M. and Bonting, S.L. (in the press) Rat pancreas adenylate cyclase. VII. Effect of extracellular calcium on pancreozymin-induced cyclic AMP formation. Biochim.Biophys.Acta.
Ridderstap, A.S. and Bonting, S.L. (1969) Na^+-K^+ activated ATPase and exocrine pancreatic secretion in vitro. Amer.J.Physiol. 217, 1721-1727.
Robberecht, P. and Christophe, J. (1971) Secretion of hydrolases by perfused fragments of rat pancreas: effects of calcium. Amer. J.Physiol. 220, 911-917.
Robberecht, P., Deschodt-Lanckman, M., De Neef, M., Borgeat, P. and Christophe, J. (1974) In vivo effects of pancreozymin, secretin, vasoactive intestinal polypeptide and pilocarpine on the levels of cyclic AMP and cyclic GMP in the rat pancreas. FEBS Lett. 43, 139-143.
Robberecht, P., Conlon, T. and Gardner, J.D. (1976) Interaction of porcine vasoactive intestinal peptide with dispersed pancreatic acinar cells from guinea pig: structural requirements for effects of VIP and secretin on cellular cyclic AMP. J.Biol.Chem. 251, 4635-4639.
Robberecht, P., Deschodt-Lanckman, M., Morgat, J. and Christophe, J. (1978a) The interaction of caerulein with the rat pancreas. 3. Structural requirements for in vitro binding of caerulein-like peptides and its relationship to increased calcium outflux, adenylate cyclase activation, and secretion. Eur.J.Biochem. 91, 39-48.
Robberecht, P., Deschodt-Lanckman, M., Van der Haegen, J.J. (1978b) Demonstration of biological activity of brain gastrin-like peptide material in human: its relationship with the COOH-terminal octapeptide of cholecystokinin. Proc.Nat.Acad.Sci. USA 75, 524-527.

Robberecht, P., Deschodt-Lanckman, M., Woussen-Colle, M.C. and Christophe, J. (1979) Interference of butyrylated,derivatives of cyclic GMP with the biological and immunological properties of the cholecystokinin gastrin family of peptides. Danish Med. Bull. 26, suppl. 1, p 1.
Robison, G.A., Butcher, R.W. and Sutherland, E.W. (1971) Cyclic AMP. Academic Press, New York.
Rochette-Egly,C., Launay, J.F. and Grenier, J.F. (1980) Implication of microtubules and microfilaments in the cyclic GMP response of rat exocrine pancreas secretagogues. FEBS Lett. 113, 335-339.
Rothman, S.S. and Brooks, F.P. (1965) Pancreatic secretion in vitro in "Cl-free", "CO_2-free" and low Na^+-environment. Amer.J.Physiol. 209, 790-796.
Rothman, S.S. (1967) "Non-parallel transport" of enzyme protein by the pancreas. Nature 213, 460-462.
Rothman, S.S. (1975) Protein transport by the pancreas. Science 190, 747-753.
Rutten, W.J., De Pont, J.J.H.H.M. and Bonting, S.L. (1972) Adenylate cyclase in the rat pancreas. Properties and stimulation by hormones. Biochim.Biophys.Acta 274, 201-213.
Rutten, W.J. (1974) On stimulus-secretion coupling in the exocrine pancreas. Thesis, University of Nijmegen, The Netherlands.
Said, S.I. and Mutt, V. (1970) Polypeptide with broad biological activity: isolation from small intestine. Science 169, 1217-1218.
Said, S.I. and Mutt, V. (1972) Isolation from porcine intestinal wall of a vasoactive octacosapeptide related to secretin and to glucagon. Eur.J.Biochem. 28, 199-204.
Salomon, Y., Londos, C. and Rodbell, M. (1974) A highly sensitive adenylate cyclase assay. Anal.Biochem. 58, 541-548.
Sanders, H. (1967) Preparative isolation of phosphatidyl serine from brain. Biochim.Biophys.Acta 144, 485-487.
Schliwa, M. (1976) The role of divalent cations in the regulation of microtubule assembly. In vivo studies on microtubules of the heliozoan axopodium using the ionophore A23187. J.Cell.Biol. 70, 527-540.
Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1975) Role of calcium in exocrine pancreatic secretion. I. Calcium movements in the rabbit pancreas. Biochim.Biophys. Acta 404, 257-267.
Schreurs, V.V.A.M. (1976) Role of calcium in exocrine pancreatic secretion. Thesis, University of Nijmegen. The Netherlands.
Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1976a) Role of calcium in exocrine pancreatic secretion. II. Comparison of the effects of carbachol and the ionophore A23187 on enzyme secretion and calcium movements in rabbit pancreas. Biochim.Biophys.Acta 419, 320-330.
Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1976b) Role of calcium in exocrine pancreatic secretion. III. Comparison of calcium and magnesium movements in rabbit pancreas. Biochim.Biophys.Acta 436, 664-674.
Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting,

S.L. (1979) Role of calcium in exocrine pancreatic secretion. V.Exchangeability of the secretory calcium pool. Biochim.Biophys. Acta 583, 208-217.
Schulz, I., Yamagata, A. and Weske, M. (1969) Micropuncture studies on the pancreas of the rabbit. Pflügers Arch. 308, 277-290.
Schulz, I., Ströver, F. and Kasprik, B. (1971a) Influence of bicarbonate-CO_2-and glycodiazine buffer on the secretion of the isolated cat's pancreas. Pflügers Arch. 329, 283-286.
Schulz, I., Ströver, F. and Ullrich, K.J. (1971b) Lipid soluble weak organic acid buffers as "substrate" for pancreatic secretion. Pflügers Arch. 323, 121-140.
Schulz, I., Kondo, S., Sachs, G. and Milutinovic, S. (1977) The role of Ca^{2+} in pancreatic enzyme secretion. In: Hormone receptors in digestive tract physiology. S.Bonfils, P.Fromageot and G. Rosselin, eds. North-Holland Publishing Company, Amsterdam. pp 275-288.
Scratcherd, T. (1974) The action of inhibitors and stimulators of phosphodiesterase and adenylate cyclase on electrolyte and enzyme secretion by the pancreas. In: Secretory mechanisms of exocrine glands. N.A.Thorn and O.H.Petersen, eds. New York Academic Press, pp 379-387.
Sewell, W.A. and Young, J.A. (1975) Secretion of electrolytes by the pancreas of the anaesthetized rat. J.Physiol. 252, 379-396.
Seybold, J., Bieger, W. and Kern, H.F. (1975) Studies on intracellular transport of secretory proteins in the rat exocrine pancreas. II. Inhibition by antimicrotubular agents. Virchows. Arch.A.Pathol.Anatom. and Histol. 368, 309-327.
Shelby, H.T., Gross, L.P., Lichty, P. and Gardner, J.D. (1976) Action of cholecystokinin and cholinergic agents on membrane-bound calcium in dispersed pancreatic acinar cells. J.Clin.Invest. 58, 1482-1493.
Siekevitz, P. and Palade, G.E. (1960) A cytochemical study on the pancreas of the guinea pig. J.Biophys.Biochem.Cytol. 7, 619-630.
Sjöstrand, F.S. (1962) The fine structure of the exocrine pancreas cell. In: Ciba Found. Symp. The exocrine pancreas. A.V.S.de Reuck, M.P. Cameron, eds. Churchill, London. pp 1-19.
Smith, P.A. and Case, R.M. (1975) Effects of cholera toxin on cyclic adenosine 3',5'-monophosphate concentration and secretory processes in the exocrine pancreas. Biochim.Biophys.Acta 399, 277-290.
Stening, G.F. and Grossman, M.I. (1969) Gastrin-related peptides as stimulants of pancreatic and gastric secretion. Amer.J.Physiol. 217, 262-266.
Stock, C., Launay, J.F., Grenier, J.F. and Bauduin, H. (1978) Pancreatic acinar cell changes induced by caerulein, vinblastine, deuterium oxide, and cytochalasin B in vitro. Lab.Invest. 38, 157-164.
Stolc, V. (1979) Control of adenylate cyclase by divalent cations and agonists. Analysis of interactions by the Hill equation. Biochim.Biophys.Acta 569, 267-276.
Stolze, H. and Schulz, I. (1980) Effect of atropine, ouabain, antimycin A, and A23187 on "trigger Ca^{2+} pool" in exocrine pancreas.

Amer.J.Physiol. 238, G338-G348.
Svoboda, M., Robberecht, P., Camus, J., Deschodt-Lanckman, M. and Christophe, J. (1976) Subcellular distribution and response to gastrointestinal hormones of adenylate cyclase in the rat pancreas. Eur.J.Biochem. 69, 185-193.
Svoboda, M., Robberecht, P., Camus, J., Deschodt-Lanckman, M. and Christophe, J. (1978) Association of binding sites for guanine nucleotides with adenylate cyclase activation in rat pancreatic plasma membranes. Interaction of gastrointestinal hormones. Eur.J.Biochem. 83, 287-297.
Swanson, C.H. and Solomon, A.K. (1973) A micropuncture investigation of the whole tissue mechanism of electrolyte secretion by the in vitro rabbit pancreas. J.Gen.Physiol. 62, 407-429.
Swanson, C.H. and Solomon, A.K. (1975) Micropuncture analysis of the cellular mechanism of electrolyte secretion by the in vitro rabbit pancreas. J.Gen.Physiol. 65, 22-45.
Tartakoff, A.M., Greene, L.J. and Palade, G.E. (1974) Studies on the guinea pig pancreas. Fractionation and partial characterization of exocrine proteins. J.Biol.Chem. 249, 7420-7431.
Theoharides, Th.C. and Douglas, W.W. (1978) Secretion in mast cells induced by calcium entrapped within phospholipid vesicles. Science 201, 1143-1145.
Tracey, H.J. and Gregory, R.A. (1964) Physiological properties of a series of synthetic peptides structurally related to gastrin I. Nature 204, 935-938.
Tyrell, D.A., Heath, T.D., Colley, C.M. and Ryman, B.E. (1976) New aspects of liposomes. Biochim.Biophys.Acta 457, 259-302.
VanderMeers, A., VanderMeers-Piret, M., Rathé, J., Kutzner, R., Delforge, A. and Christophe, J. (1977) A calcium-dependent protein activator of guanosine 3':5'-monophosphate phosphodiesterase in bovine and rat pancreas. Isolation, properties and levels in vivo. Eur.J.Biochem. 81, 379-386.
Van Nispen, J.W. (1974) Synthesis and properties of some ACTH and α-MSH analogues. Structure-function relation. Thesis, University of Nijmegen. Nijmegen, The Netherlands.
Van Nispen, J.W., Smeets, P.J.H., Poll, E.H.A. and Tesser, G.I. (1977a) Investigation of the role of tryptophan in α-MSH. Replacement by L-Pentametylphenylalanine and L-phenylalanine. Int.J.Pept.Prot.Res. 9, 203-212.
Van Nispen, J.W., Tesser, G.I., Barthe, P.L., Maier, R. and Schenkel-Holliger, L. (1977b) Biological activities of ACTH-analogues varied in the active site. Acta Endocrinol. 84, 470-484.
Wang, C.C. and Grossman, M.I. (1951) Physiological determination of the release of secretin and pancreozymin from intestine of dogs with transplanted pancreas. Amer.J.Physiol. 164, 527-545.
Weinstein, J.N., Yoshikama, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Liposome-cell interaction: transfer and intracellular release of a trapped fluorescent marker. Science 195, 489-492.
Weinstein, J.N., Magin, R.L., Yatvin, M.B. and Zaharko, D.S. (1979) Liposomes and local hyperthermia: selective delivery of methotrexate to heated tumors. Science 204, 188-191.

Williams,J.A. and Lee,M.(1974) Pancreatic acinar cells:use of a Ca^{2+}-ionophore to separate enzyme release from the earlier steps in stimulus-secretion coupling.Biochem.Biophys.Res.commun.60,542-548.
Williams, J.A. (1975) Na^{+} dependence of in vitro pancreatic amylase release. Amer.J.Physiol. 229, 1023-1026.
Williams, J.A. and Lee, M. (1976) Microtubules and pancreatic amylase release by mouse pancreas in vitro. J.Cell.Biol. 71, 795-806.
Williams, J.A., Cary, P. and Moffat, B. (1976) Effects of ions on amylase release by dissociated pancreatic acinar cells. Amer.J. Physiol. 231, 1562-1567.
Williams, J.A. (1978) The effect of the ionophore A23187 on amylase release, cellular integrity and ultrastructure of mouse pancreatic acini. Cell Tiss.Res. 186, 287-295.
Wilson, R.M., Boden, G., Shore, L.S. and Essa-Koumar, N. (1977) Effect of somatostatin on meal-stimulated pancreatic exocrine secretions in dogs. Diabetes 26, 7-10.
Yabe, Y., Miura, C., Horikoski, H. and Baba, Y. (1976) Analogues of luteinizing hormone-releasing hormone with modification in position 3. Chem.Pharm.Bull. 24, 3149-3157.
Yabe, Y., Morita, A., Miura, C., Kobayashi, S. and Baba, Y. (1977) Synthesis and biological activity of tetragastrin analogues modifying the tryptophan residue. Chem.Pharm.Bull. 25, 2731-2734.
Yajima, H., Mori, Y., Koyoma, K., Tobe, T., Setoyama, M., Adachi, H., Kanno, T. and Saito, A. (1976) Studies on peptides LXVIII. Synthesis of the tritriacontapeptide amide corresponding to the entire amino acid sequence of the desulfated form of porcine cholecystokinin-pancreozymin (CCK-PZ). Chem.Pharm.Bull. 24, 2794-2802.

CURRICULUM VITAE

Bernadet Renckens werd op 3 november 1948 geboren te Hoorn (N. H.). In 1968 behaalde zij het diploma gymnasium-β aan het Sint Werenfridus Lyceum te Hoorn. In hetzelfde jaar begon ze met de studie scheikunde aan de Katholieke Universiteit te Nijmegen. In 1971 legde ze het kandidaatsexamen (s2) af en in 1975 het doctoraalexamen (hoofdrichting biochemie, bijvakken chemische microbiologie en fysiologie). Van augustus 1974 tot augustus 1976 was zij lerares scheien natuurkunde aan de Scholengemeenschap Nijmegen-West. Van augustus 1976 tot juni 1980 was zij als wetenschappelijk medewerkster verbonden aan het Laboratorium voor Biochemie. In deze periode heeft zij het hier beschreven onderzoek uitgevoerd en daarnaast een bijdrage geleverd aan het onderwijs voor medische en tandheelkundige studenten.
In 1974 is zij getrouwd met Jan Tuinte.

STELLINGEN

1. Om een juist inzicht te krijgen in de rol van calciumionen bij een bepaald fysiologisch proces, is het noodzakelijk naast $^{45}Ca^{2+}$-fluxen ook het totaal cellulair calciumgehalte te meten.

   Dit proefschrift.

2. De remming van de gestimuleerde enzymsecretie in de pancreas door verlaging van de extracellulaire natrium concentratie is niet het gevolg van een vermindering van de vloeistofsecretie.

   Dit proefschrift.

3. Het model voor de acineuze vloeistofsecretie in de pancreas, opgesteld door Putney, is onvolledig, omdat het geen rekening houdt met de bijdrage van de paracellulaire weg aan dit proces.

   Putney, J.W. (1979) Pharmacol. Rev. 30 209-245.

4. De door Sen en Ray gegeven molaire verhouding tussen fosfolipiden en cholesterol in de microsomale fractie van de varkensmaagmucosa is niet in overeenstemming met de door hen gegeven gehalten van deze lipiden.

   Sen, P.C. en Ray, T.K. (1979)
   Arch. Biochem. Biophys. 198 548-555

   Sen, P.C. en Ray, T.K. (1980)
   Arch. Biochem. Biophys. 202 8-17

5. Het gebruik van gestandaardizeerde zuurselconcentraten bij de zuivelbereiding gaat ten koste van het eigen karakter van het product.

   Jaarverslag 1979,
   Nederlands Instituut voor Zuivelonderzoek.

6. Het kiemgetal is een onvoldoende parameter voor de bacteriologische kwaliteit van melk bestemd voor de bereiding van boerenkaas.

7. Het werkzame effect van de fistelpot, bereid volgens het recept van de familie Ursem, berust waarschijnlijk op de aanwezigheid van een antibiotische component.

   Molenaar, K. (1978) West-Frieslands Oud en Nieuw 70-80 (uitgever: Historisch genootschap "Oud West-Friesland").

8. Gezien de verwachtingen die nu al tien jaar onder medische analisten worden gewekt, verdraagt de totstandkoming van de wet op de medische analisten geen verder uitstel meer.

9. Het geven van reiskostenvergoedingen aan rijksambtenaren in het woon-werkverkeer gedurende onbeperkte tijd is in strijd met het streven van de regering naar vermindering van de groei van de mobiliteit.

   Derde nota over de ruimtelijke ordening, Verstedelijkingsnota (1979).

10. Het is een gezonder uitgangspunt om in openbare ruimten aan te geven waar wel, in plaats van, zoals nu gebruikelijk is, waar niet mag worden gerookt.

11. Het is een wijdverbreid misverstand dat producten van natuurlijke oorsprong altijd gezonder c.q. minder schadelijk zouden zijn dan vergelijkbare synthetische producten.

12 september 1980 Bernadet Renckens